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PARA SU PRESENTACION

Dr/a. **Maria Dolores Boyano Lopez**

como Tutor/a de la Tesis Doctoral: **Lipidomics in Melanoma: Identification of new lipid prognostic biomarkers**

realizada en el Programa de Doctorado **Investigación biomédica**

por el Doctorando Don/ña. **Arantza Perez Valle**

y dirigida por el Dr./a **Aintzane Asumendi Mallea**

autorizo la presentación de la citada Tesis Doctoral, dado que reúne las condiciones necesarias para su defensa.

En Leioa a 04 de Febrero de 2020

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La Comisión Académica del Programa de Doctorado en **Investigación Biomédica**

en reunión celebrada el día 12 de FEBRERO de 2020, ha acordado dar la conformidad a la presentación de la Tesis Doctoral titulada: **Lipidomics in Melanoma: Identification of new lipid prognostic biomarkers**

dirigida por el/la Dr/a. **Maria Dolores Boyano Lopez y la Dra. Aintzane Asumendi Mallea**

y presentada por Don/Dña. **Arantza Perez Valle**

adscrito o adscrita al Departamento **Biología celular e histología**

En Leioa a 12 de febrero de 2020

EL/LA RESPONSABLE DEL PROGRAMA DE DOCTORADO

Fdo.: M. B. Ruiz-Larrea

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El Consejo del **Departamento de Biología Celular e Histología**

en reunión celebrada el día 23 de ENERO de 2020 ha acordado dar la conformidad a la admisión a trámite de presentación de la Tesis Doctoral titulada: **Lipidomics in Melanoma: Identification of new lipid prognostic biomarkers**

dirigida por el/la **Dra. Maria Dolores Boyano Lopez y la Dra. Aintzane Asumendi Mallea**

y presentada por Don/ña. **Arantza Perez Valle**

ante este Departamento.

En LEIOA a 23 de ENERO de 2020

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TITULO DE LA TESIS: **Lipidomics in Melanoma: Identification of new lipid prognostic biomarkers**

El Tribunal designado por la Comisión de Postgrado de la UPV/EHU para calificar la Tesis Doctoral arriba indicada y reunido en el día de la fecha, una vez efectuada la defensa por el/la doctorando/a y contestadas las objeciones y/o sugerencias que se le han formulado, ha otorgado por_____la calificación de:

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DOCTORAL THESIS

LIPIDOMICS IN MELANOMA: IDENTIFICATION OF NEW LIPID PROGNOSTIC BIOMARKERS

Arantza Perez Valle

Cell Biology and Histology department

Faculty of Medicine and Nursing

Leioa, 2020

This doctoral thesis has been conducted thanks to the funding by:

1. Desarrollo del Sistema ONCOFINDER para la detección y prognosis de tumores mediante biomarcadores lipídicos utilizando microarrays de membranas. Ministry of Economy, Industry and Competitiveness of Spain. 2015-2018 (RTC-2015-3693-1).
2. Movilidad y difusión de los resultados de la investigación en la UPV/EHU. Scholarship provided by UPV/EHU. 2018.
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5. Melamics. Investigación Multidisciplinar del Melanoma Maligno. Identificación de nuevos biomarcadores de utilidad en el pronóstico y tratamiento del melanoma. Department of Economic Development and Infrastructures of the Basque Government. 2016-2017. (KK2016-00036; KK2017-00041).

Some of the results obtained in this work have been object of patenting:

- Oncofinder: A novel platform for screening benignant nevi from melanomas based on lipid phenotype using mass spectrometry and machine learning.

The results of this thesis have been published in the following works:

1. Influence of lipid fragmentation in the data analysis of imaging mass spectrometry experiments. J. Garate, S. Lage, L. Martín-Saiz; A. Perez-Valle, B. Ochoa, M.D. Boyano, R. Fernández, J.A. Fernández. Journal of the American Society for Mass Spectrometry Experiments. 2020.
2. Lipidomics Biomarkers for Melanoma. A. Perez-Valle, J. Garate, R. Fernandez, S. Lage, E. Astigarraga, G. Barreda-Gomez, J. Fernandez, A. Asumendi, B. Ochoa, M.D. Boyano. Journal of investigative Dermatology.137(10).289.2017
3. Fosfolipasa D2 entzimaren garrantzia melanomaren sorreran eta garapenean. A. Perez-Valle, M.D. Boyano, A. Asumendi. Osagaiz. 2. Bolumena. 2. Ale berezia. 13(52). 2018

The results of this thesis have been presented in the following congresses:

1. Lipidic biomarkers in melanoma. 47th annual ESDR meeting. Salzbourg, Austria. 2017. Winner of the best poster presentation.
2. Comparative lipidomic study of normal skin melanocytes, nevus melanocytes and melanoma cells. SEBC Joint Congress. Gijon, Spain. 2017.
3. Fosfolipasa D2 entzimaren garrantzia melanomaren sorreran eta garapenean. Osasun-zientzietako ikertzaileen III. Topaketak. Eibar, Spain. 2018.
4. Identificación de nuevos biomarcadores en melanoma. III Reunión de Investigación translacional en melanoma. Iruña-Pamplona, Spain. 2018.
5. Melanomaren biomarkatzaile lipidikoak. II. Ikergazte. Nazioarteko ikerketa euskaraz. Iruña-Pamplona, Spain. 2017.
6. These results have been presented in five other international congresses by other authors of the research group.

ESKER ONAK

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SUMMARY

Melanoma is the cancer that arises upon the malignant transition of melanocytes and, even though it can occur in different locations of the body, it preferentially appears on the skin. In this way, it is representative of 4% of all skin tumors; however, 80% of deaths from skin cancer are due to melanoma. Moreover, the incidence rates of melanoma have steadily increased in recent decades, and it is the seventh most diagnosed cancer in Europe.

Melanoma tumors are very heterogeneous, which has complicated the finding of an efficient biomarker or therapy for this cancer. As a matter of fact, in the present, there is no specific biomarker or effective therapy for melanoma. It is considered a very aggressive tumor and metastasizes easily; thus, if it is not diagnosed early, survival rates drop dramatically. It stands to reason that there is a great need to identify new biomarkers to improve the early detection, diagnosis and prognosis of melanoma, in addition to finding new therapeutic targets.

In this regard, cellular metabolism has gained attention in cancer research recently. Indeed, the disruption of cancer metabolism has been established as a hallmark of cancer. A large body of work demonstrates that cancer cells undergo metabolic rewiring that supports the augmentation in cellular activities that guarantees their malignant phenotype. It is known that the variations in metabolic pathways within cells alter the amount and composition of some lipid species. In particular, alterations in lipid metabolism of cancer cells have been linked to increased proliferation and metastasis, reduction of cell death and resistance to therapy, among other issues. Most lipidomic studies in cancer have mainly focused in breast, colon, lung and prostate cancers, but unfortunately, little is known about melanoma's lipidome. Therefore, we hypothesize that, like in other types of cancer, the malignant transformation of healthy melanocytes into tumor cells might be favored by the adaptation of their metabolism and, thereby, the amount and composition their lipid content. Hence, the principal objective of this thesis was to identify new lipid biomarkers by comparing the lipidome of non-pathological melanocytes and malignant melanomas to detect the particular lipid species that drive the differentiation between these tumor and non-pathological cells. In addition, a secondary objective was to determine the biological effects that these alterations in the lipid content could have on cells.

For biomarker research, the lipidome of skin and nevus melanocyte cell lines, and primary and metastatic melanoma cell lines were studied using different lipidomic approaches. Lipidomic analyses enable the classification of tumor and healthy tissues by comparing the lipid fingerprint of the samples and the interactions of those lipids with other lipids, proteins, metabolites and genetic material. Most lipidomic analyses are based on mass spectrometry. In this work, two different lipidomic strategies have been carried out. First, lipid extracts of several skin and nevus melanocytes, and primary and metastatic melanoma cell lines were obtained using Bligh & Dyer methodology. The extraction method used hampered the extraction of all lipid classes, and the most polar molecular species were not extracted. The obtained lipid extracts were studied using an UHPLC-ESI-MS/MS mass spectrometry approach. This allowed a global lipidomic study, which confirmed that malignant and non-malignant cells might have a different lipid profile. Besides the global lipidome of the cells, a deeper insight into the results revealed that we could detect differences in the intensities of the lipid species that make up the various lipid families. Specifically, we found that a panel of 45 lipid species presents significantly altered levels that allows classifying healthy and malignant cells. The detected alterations in lipid content are proposed to support the malignant phenotype of cancer cells.

The results obtained with the first lipidomic approach confirmed that there are particular lipid species that have a differential presence in healthy and malignant cells, and these lipids

pertained to the lipid subclasses that are mainly located in the cell membranes. For this reason, the following lipidomic strategy was performed on functional cell membrane microarrays, a promising biotechnological tool with translational potential. Here, functional cell membranes of the studied cell lines were immobilized, and then a MALDI-MS lipidomic approach was applied. This is a useful tool, as a small amount of each sample is needed, the preparation of the samples is fast and reproducible, no lipid extraction is needed, and MALDI-MS is the gold standard method for biomarker discovery. After performing different statistical analyzes, we could detect 116 species of lipids that show a significantly altered intensity in healthy melanocytes and malignant melanoma cell lines. In particular, there are 48 and 54 lipid species with a differential levels between skin or nevus melanocytes and primary melanomas, respectively. The difference between the lipid content of skin/nevus melanocytes and metastatic melanoma is also evident, since there are 82 molecules that are potential biomarkers to differentiate between skin melanocytes and metastatic melanomas, and 81 lipid species in the comparison of nevus melanocytes and metastatic melanomas cell lines. Moreover, with this analytical approach we were able to detect 11 lipid species that exhibited differential intensities between skin and nevus melanocytes. Interestingly, three lipid species show differential levels in primary and metastatic melanoma, and, although further research is needed, they could be useful prognostic markers.

Lipidomic analyses demonstrated that phospholipids have an altered presence in melanoma cells compared to non-transformed melanocytes. These lipids are metabolized by phospholipase enzymes, which have been previously described to exhibit altered expression and activity in different cancers. Hence, we studied the expression levels of this family of enzymes in melanomas and found that PLD2 has upregulated protein expression and activity in melanoma cells compared to skin melanocytes. PLD2 metabolizes phosphatidylcholines (PC), which is consisted with the results obtained in the lipidomic analyses, since PCs have also been detected with greater intensity in melanoma cells compared to normal melanocytes. Therefore, we hypothesize that PLD2 plays a role in some of the processes involved in melanoma development and metastatic dissemination *in vitro*. To study the particular implication of PLD2 in the carcinogenic process, this enzyme was overexpressed and silenced in various primary and metastatic melanoma cell lines. The results showed that the augmented activity and expression of this enzyme significantly increases the proliferation, migration and invasion of these cells, while the downregulation of PLD2 reduces these processes. Thus, PLD2 seems to be involved in melanoma development and progression, and its blockade could be a promising therapeutic strategy.

LABURPENA

Melanoma melanozitoen gaiztotzearen ondorioz sortzen den minbizia da eta, gorputzaren hainbat tokitan gerta daitekeen arren, nagusiki larruazalean agertu ohi da. Izan ere, larruazalean ematen diren tumore guztien %4 melanoma dira; aldiz, horien ondorioz ematen diren heriotzen %80aren erantzule da. Gainera, melanomaren intzidentzia tasak etengabe gora egin du azken hamarkadetan, eta Europako zazpigarren minbizi diagnostikatuena da.

Melanoma tumoreak oso heterogeneoak dira, eta horrek asko zaildu du biomarkatzaile edo terapia eraginkor bat aurkitzea. Hortaz, gaur egun ez dago biomarkatzaile edo terapia zehatzik melanomarako, batez ere, melanoma metastatikorako. Oso tumore oldarkorra da eta erraz sortzen ditu metastasiak; beraz, ez bada garaiz diagnostikatzen, biziraupen-tasak behera egiten du nabarmenki. Horregatik, melanomaren antzemate goiztiarra, diagnostikoa eta pronostikoa hobetuko luketen biomarkatzaile berriak identifikatzea ezinbestekoa suertatzen da.

Metabolismo zelularrak garrantzia irabazi du azken urteotan minbiziaren ikerketan. Izan ere, minbizi zeluletan ematen den bidezidor metabolikoen eraldaketa minbiziaren bereizgarri gisa ezarri da. Aldaketa metaboliko horiek, minbizi zelulen fenotipo gaiztoa bermatzen dituzten zelula-jarduerak sustatzen dituzte. Jakina da zelulen bidezidor metabolikoen aldaketek lipido-espezie batzuen kantitatea eta konposaketa aldatzen dituztela. Zehazki, minbizi-zeluletako lipidoen metabolismoan ematen diren alterazioek zelulen hazkuntzan eta metastasian eragiten dute, eta aldi berean, zelulen heriotza murriztu eta terapiarekiko erresistentzia sortzen dute, besteak beste. Minbiziaren ikerketan burutu diren analisi lipidomiko gehienak bularreko, koloneko, biriketako eta prostatako minbizietan izan dira, baina, zoritxarrez, ezer gutxi dakigu melanomaren lipidomari buruz. Beraz, gure **hipotesia** ondokoa da: beste minbizi mota batzuetan bezala, metabolismoaren egokitzapenak, eta beraz, lipido edukiaren aldaketek, melanozito osasuntsuen eraldaketa gaiztoa bultzatzen dutela. Hori dela eta, tesiaren **helburu nagusia** lipido biomarkatzaile berriak identifikatzea izan zen, larruazaleko melanozitoen eta melanoma gaiztoen lipido edukia alderatuz, tumore horien eta zelula ez patologikoen arteko desberdintasuna gidatzen duten espezie lipidiko partikularrak hautemateko. Bigarren helburua, lipidoen edukian sortutako alterazio horiek zeluletan izan ditzaketen eragin biologikoa zehaztea izan zen.

Biomarkatzaileak ikertzeko, larruazaleko melanozitoen, nevuseko melanozitoen, melanoma primarioen eta melanoma metastatikoen lipidomak aztertu ziren. Horretarako tumore eta ehun osasuntsuak sailkatzea ahalbidetzen duten hainbat teknika lipidomiko erabili ziren. Analisi lipidomikoek laginen lipido edukia eta lipido horiek beste lipido, proteina, metabolito eta material genetikoarekin duten elkarreraginak aztertzen dituzte eta gehienak masa espektrometrian oinarritzen dira. Lan honetan, bi estrategia lipidomiko desberdin erabili dira. Lehenik, larruazaleko eta nevuseko melanozitoetatik, melanoma primario eta metastatikoetatik lipido erauzketa egin zen Bligh & Dyer metodologia erabiliz. Metodo horrekin ezin izan ziren lipido mota guztiak lortu, lipido polarrenak deuseztatu baitziren. Lipido erauzkin horiek UHPLC-ESI-MS/MS masa espektrometria estrategia erabiliz aztertu ziren. Horrek, azterketa lipidomiko orokor bat ahalbidetu zuen, zeinak zelula gaizto eta osasuntsuen lipido profila ezberdina dela baieztatu zuen. Zelulen lipidoma orokorraz gain, emaitzen ikuspegi sakonago batek agerian utzi zuen lipido familia ezberdinak osatzen dituzten lipido espezie konkretuen artean intentsitate desberdintasunak antzeman genitzakeela. Zehazki, zelula osasuntsu eta gaiztoetan intentsitate mailak esanguratsuki ezberdinak dituzten 45 lipido espeziez osatutako multzo bat aurkitu genuen. Lipidoen edukian antzemandako alterazioek minbizi-zelulen fenotipo gaiztoasostengatzen dutela proposatzen da.

Laburpena

Aipaturiko estrategia lipidomikoarekin baieztatu genuen zelula osasuntsu eta gaiztoetan presentzia diferentziala duten espezie lipidiko partikularrak daudela, eta aurkitutako lipido horiek batez ere zelula mintzetan kokatzen diren lipido familien osagaiak direla. Horregatik, hurrengo teknika lipidomikoa mintz zelular funtzionalez osatutako mikroarraiak erabiliz burutu zen. Hemen, aztertutako lerro zelularren mintz-esekidurak jarri ziren eta MALDI-MS teknika lipidomikoa erabili. Hori, translazio-potentziala duen tresna erabilgarria da; izan ere, lagin bakoitzaren kantitate txiki bat nahiko da, laginen prestaketa azkarra eta errepoduziblea da, ez da lipido erauzketarik behar, eta MALDI-MS teknika biomarkatzaileak aurkitzeko metodo estandarra da. Analisi estatistiko ezberdinak burutu ondoren, melanozito osasuntsuetan eta melanoma gaiztoaren lerro zelularretan intentsitate nabarmen ezberdina erakusten duten 116 lipido espezie daudela aurkitu zen. Konkretuki, adierazpen diferentzial duten 48 eta 54 lipido espezie daude larruazaleko edo nevuseko melanozitoen eta melanoma primarioen artean, hurrenez hurren. Larruazaleko edo nevuseko melanozitoen eta melanoma metastatikoen lipido edukiaren arteko aldea agerikoa da ere. Izan ere, 82 lipido-espezie aurkitu dira larruazaleko melanozitoak eta melanoma metastatikoak bereizteko biomarkatzaile potentzialak izan daitezkeenak, eta 81 lipido espezie nevuseko melanozitoak eta melanoma metastatikoen arteko konparaketan. Gainera, ikuspegi analitiko horrek, larruazaleko eta nevuseko melanozitoen artean intentsitate diferentzialak dituzten 11 lipido-espezieak hautematea ahalbidetu zuen. Bestalde, hiru lipido espezie detektatu dira pronostiko markatzaile bezala erabilgarriak izan daitezkeenak, melanoma primarioan eta metastatikoan maila diferentzial nabarmenak dituztenak alegia.

Analisi lipidomikoek fosfolipidoak melanoma zeluletan bestelako presentzia dutela frogatu zuten. Lipido horiek fosfolipasa entzima-familiako kideen bitartez metabolizatzen dira. Aurretik, entzima horiek hainbat minbizietan euren proteina adierazpena eta jarduera aldatzen dutela deskribatu da. Beraz, entzima-familia horren proteina adierazpen mailak aztertu genituen melanozito eta melanoma lerro zelularretan. Emaitzak erakutsi zutenez, PLD2-ren adierazpena eta jarduera handitua dago ere melanoma zeluletan. PLD2-k fosfatidilkolinak (PC) metabolizatzen ditu, eta hori bat dator analisi lipidomikoetan lortutako emaitzekin, PC-ak melanoma zeluletan intentsitate handiagoz detektatu baitira melanozitoekin alderatuta. Beraz, PLD2-k melanomaren garapenean eta metastasian parte hartzen duten hainbat prozesutan eragina duela proposatu da. PLD2-k prozesu kantzerigenoan duen inplikazio partikularra aztertzeko, entzima hori hainbat melanoma primario eta metastatiko lerro zelularretan gain-adierazi eta isildu zen. Emaitzek erakutsi zuten entzima horren jarduera eta adierazpena handitzeak nabarmenki areagotzen dutela zelula horien hazkuntza, migrazioa eta inbasioa; PLD2-ren isilerak, berriz, prozesu horiek murrizten ditu. Beraz, PLD2-a melanomaren garapenean eta progresioan parte hartzen duela antzematen da, eta hortaz, bere blokeoa etorkizun handiko estrategia terapeutikoa izan liteke.

LIPIDOMICS IN MELANOMA: IDENTIFICATION OF NEW LIPID PROGNOSTIC BIOMARKERS

Introduction

1. Melanoma 1
1.1. Epidemiology 1
1.1.1. Incidence 1
1.1.2. Mortality 3
1.2. Etiology 4
1.2.1. Intrinsic factors 4
1.2.2. Extrinsic factor 5
1.3. Histopathology 6
1.3.1. Skin 6
1.3.2. Melanoma development 8
1.3.3. Melanoma diagnosis and staging 9
1.3.4. Clinical classification of melanoma 15
1.4. Melanoma treatment 16
1.4.1. Metastatic melanoma 16
1.4.2. New challenges 17
1.5. Melanoma biomarkers 18
1.5.1. Biomarker discovery 19
1.6. Melanoma hallmarks 19
2. Lipids, cell metabolism and cancer 21
2.1. Lipids 21
2.1.1. Lipid classification 21
2.1.2. Lipid nomenclature 35
2.2. Lipidomic analyses 37
2.2.1. UHPLC-ESI-MS/MS methodology 37
2.2.2. MALDI-MS methodology 40
2.3. Cancer metabolism 41
2.3.1. Lipid metabolism and cancer 42
3. Phospholipase D and cancer 46

Hypothesis & Objectives

1. Hypothesis 53
2. Objectives 54

Materials & Methods

1. Materials 57
1.1. Reactants ... 57
1.2. Commercial cell lines ... 58
2. Methods 59
2.1. Cell culture ... 59
2.1.1. Defrosting cells ... 60
2.1.2. Cell subculture ... 60
2.1.3. Freezing cells ... 61
2.1.4. Mycoplasma detection ... 61
2.1.5. Cell pellet collection ... 61
2.1.6. Cell transfection ... 61
2.1.7. Cell proliferation assay ... 63
2.1.8. Cell invasion assay ... 63
2.1.9. Cell migration assay ... 64
2.2. Melanocyte isolation from human nevus ... 65
2.3. Cellular lipids analysis ... 66
2.3.1. Sample homogenization ... 66
2.3.2. Protein quantification ... 66
2.3.3. Lipid extraction ... 66
2.3.4. Lipidomic analysis ... 67
2.4. Cell membrane analysis ... 71
2.4.1. Cell membrane isolation ... 71
2.4.2. Protein quantification ... 71
2.4.3. Microarray development ... 71
2.4.4. Lipidomic analysis ... 72
2.5. Protein analysis ... 73
2.5.1. Western blot ... 73
2.5.2. Immunofluorescence ... 77
2.6. PLD enzymatic activity assay *in vitro* ... 78

Results

1. Lipidomic analysis of lipid extracts of melanocyte and melanoma cell lines 83

1.1. Differential global lipotype between melanocytes and melanoma cells 84

1.2. Different lipid classes exhibit altered levels in melanoma cells 89

1.2.1. Glycerophospholipids 89

1.2.2. Glycerolipids 95

1.2.3. Free fatty acids 96

1.2.4. Sphingolipids 97

1.2.5. Cholesterol esters 98

1.3. Lipid biomarker discovery for malignancy 99

2. Lipidomic analysis of functional membrane microarrays 117

3. Phospholipase D2 enhances melanoma progression and metastatic behavior 131

3.1. PLD2 enzyme is upregulated in melanoma cells 131

3.2. PLD2 involvement in the carcinogenic process 135

Discussion

1. Global lipid profiling of human melanocytes and melanoma cell lines 141

2. Finding new lipid biomarkers for melanoma 149

3. Phospholipase D2 promotes tumorigenic and metastatic activities in melanoma cells 155

Conclusions 161

Appendix 165

References 262

LIPIDOMIKA MELANOMAN: PRONOSTIKORAKO LIPIDO BIOMARKATZAILE BERRIEN IDENTIFIKAZIOA

Sarrera

1. Melanoma 184
1.1. Epidemiologia 184
1.1.1. Intzidentzia 184
1.1.2. Heriotza-tasa 186
1.2. Etiologia 187
1.2.1. Faktore intrintsekoak 187
1.2.2. Faktore estrintsekoak 188
1.3. Histopatologia 189
1.3.1. Larruazala 189
1.3.2. Melanoma garapena 191
1.3.3. Melanomaren diagnostikoa eta estadifikazioa 192
1.3.4. Melanomaren sailkapen klinikoa 198
1.4. Melanoma tratamenduak 199
1.4.1. Melanoma metastatikoa 199
1.4.2. Aukera terapeutiko berriak 200
1.5. Melanomaren biomarkatzaileak 201
1.5.1. Biomarkatzaile berrien aurkikuntza 202
1.6. Melanomaren ezaugarri bereizgarriak 203
2. Lipidoak, zelula-metabolismoa eta minbizia 205
2.1. Lipidoak 205
2.1.1. Lipidoen sailkapena 205
2.1.2. Lipidoen izendapena 219
2.2. Analisi lipidomikoak 220
2.2.1. UHPLC-ESI-MS/MS metodologia 221
2.2.2. MALDI-MS metodologia 224
2.3. Minbizi zelulen metabolismoa 225
2.3.1. Lipidoen metabolismoa eta minbizia 226
3. D Fosfolipasa eta minbizia 230

Hipotesia eta helburuak

1. Hipotesia 236
2. Helburuak 237

Eztabaida

1. Giza melanozito eta melanoma lerro zelular ezberdinen profil lipidiko globala 240
2. Melanomarako biomarkatzaile lipidiko berrien aurkikuntza 248
3. D2 fosfolipasak melanomaren ezaugarri protumoral eta prometastatikoak sustatzen ditu 253

Ondorioak 258

References / Erreferentziak 262

FIGURE INDEX

Figure 1. Estimated incidence rates of the diagnosed top 10 cancer types in Europe in 2018. Both sexes and all ages are considered. 1
Figure 2. Incidence of melanoma in the U.S. according to the different ethnic groups, between the years 1975-2011. 2
Figure 3. Melanoma incidence by age and sex, during 2007-2011 in the United States..... 2
Figure 4. Estimated worldwide skin melanoma incidence distribution for 2018 3
Figure 5. Fitzpatrick scale. 4
Figure 6. The principal cell types present in the epidermis 7
Figure 7. Representation of the Clark model for melanoma developmen 8
Figure 8. Examples of the ABCDE criteria for the early detection of melanoma. 10
Figure 9. Clinical description of each stage of melanoma development. 14
Figure 10. Timeline of melanoma treatment options since 2011..... 17
Figure 11. General structure of the different lipid classes..... 21
Figure 12. De novo synthesis pathways of PC, PE and PS. Adapted from..... 24
Figure 13. PI cyce..... 27
Figure 14. Glycerophospholipid biosynthesis. 30
Figure 15. Schematic representation of the general structure of sphingolipids 31
Figure 16. Representation of the metabolic pathways of sphingolipid metabolism 33
Figure 17. Schematic representation of the different components of a mass spectrometer 39
Figure 18. Workflow of tandem MS/MS strateging 40
Figure 19. Schematic representation of the signaling pathways that regulate metabolic rewiring in cancer cell..... 42
Figure 20. Schematic representation of the lipid metabolism rewiring in cancer cells..... 43
Figure 21. Cleavage sites of the different phospholipases 46
Figure 22. Workflow schema of PLD2 overexpression and silencing..... 63
Figure 23. Scheme of the transwell in vitro invasion assay, and eventual visualization of the cells that invaded 64
Figure 24. Nevus melanocyte isolation process at different steps 66
Figure 25. Injection order of the samples analyzed 68
Figure 26. Schematic representation of the PLD enzymatic assay reaction 79
Figure 27. Categorization of the lipid species identified in the UHPLC-ESI-MS/MS method..... 83
Figure 28. PCA of the study samples and QC samples in both ESI + and ESI-. 84
Figure 29. Principal components analysis of the samples in ESI+ and ESI-. 85
Figure 30. PLS-DA analysis of the samples detected in ESI+ classified into groups. 87
Figure 31. PLS-DA analysis of the samples detected in ESI- classified into groups.)..... 88
Figure 32. Comparison of the sum of the intensities of all the ethanolamine lipid species (A) and choline bearing lipid species in the four study groups. Ratio of the intensities of choline/ethanolamine lipid species in the four study groups. All the sums are related to skin melanocyte values (M = 1). 93
Figure 33. Comparison of the sum of the intensities detected for all the phosphatidylinositol species (PI), phosphatidylglycerol species (PG) and phosphatidylserine species (PS) in the four study groups, related to skin melanocyte values (M=1) 94
Figure 34. Comparison of the sum of the intensities obtained for diglycerides (DG) and triglycerides (TG) in the four study groups, related to skin melanocyte values (M=1). 95

Figure 35. Comparison of the intensities obtained for all the free fatty acid (FFA) species detected in the four study groups, related to skin melanocyte values (M=1) 96
Figure 36. A) Comparison of the sum of the intensities obtained for all the detected ceramides (Cer) in the four study groups, related to skin melanocytes (M=1). B) Comparison of intensities of short and long-ceramides, related to skin melanocytes (M=1). 97
Figure 37. Comparison of the sum of intensities obtained for all the sphingomyelin (SM) species and hexosylceramide (HexCer) species detected in the four study groups, related to skin melanocytes (M=1). 98
Figure 38. Comparison of the sum of intensities detected for all the cholesterol esters (CE) species in the four study groups, related to skin melanocytes (M=1). 98
Figure 39. Identification of the possible lipid biomarkers for healthy and malignant cells in ESI+. 100
Figure 40. Identification of the possible lipid biomarkers for healthy and malignant cells in ESI- 100
Figure 41. Identification of the potential lipid biomarkers for nevus melanocytes and primary melanoma cells in ESI+. 102
Figure 42. Identification of the potential lipid biomarkers for nevus melanocytes and primary melanoma cells in ESI-. 102
Figure 43. Identification of the potential lipid biomarkers for nevus melanocytes and metastatic melanoma cells in ESI+. 104
Figure 44. Identification of the potential lipid biomarkers for nevus melanocytes and metastatic melanoma cells in ESI-. 104
Figure 45. Identification of the potential lipid biomarkers for primary and metastatic melanoma cells in ESI-. 106
Figure 46. Box-Whisker graphs for the SMs identified as potential biomarkers for melanoma 110
Figure 47. Box-Whisker graphs for the TGs identified as potential biomarkers for melanoma. 111
Figure 48. Box-Whisker graphs for the FAs identified as potential biomarkers for melanoma. 112
Figure 49. Box-Whisker graphs for the PIs identified as potential biomarkers for melanoma. 113
Figure 50. Box-Whisker graphs for the ethanolamine lipids identified as potential biomarkers for melanoma 114
Figure 51. Box-Whisker graphs for the choline lipids identified as potential biomarkers for melanoma. 115
Figure 52. Box-Whisker graphs for the PGs identified as potential biomarkers for melanoma. 116
Figure 53. A) Schema of the sample distribution in the cell membrane microarrays. B) Clustering aggrupation of the samples in 5 clusters (Cl) and 15 clusters (Cl), representing the similarity based on the color scale 118
Figure 54. Overview of the lipid species detected in MALDI-MS and representation of their significance to differentiate melanocytes from melanoma cells. 119
Figure 55. Protein expression pattern of different phospholipases (PLA_2, PLC, PLD1, PLD2) in 3 skin melanocytes, 5 primary melanomas and 9 metastatic melanoma cell lines assessed by Western blot. 132
Figure 56. Identification of PLD2 expression pattern in skin melanocytes, primary melanoma and metastatic melanoma cell lines by immunofluorescence 133

Figure 57. PLD enzymatic activity for melanocytes, primary melanomas and metastatic melanomas. 134
Figure 58. PLD2 protein expression (A) and PLD enzymatic activity (B) studies in PLD2 overexpressed and silenced cell lines. 135
Figure 59. (A) Summary of the results obtained for PLD2 overexpressed and silenced melanoma cell lines in PLD activity, cell proliferation, migration and invasion. Graphical visualization of cell proliferation (B), migration (C) and invasion (D). studies in PLD2 overexpressed and silenced cell lines 137
Figure 60. Permutation tests for the validation of PLS-DA analysis of the samples detected in ESI+ ionization mode classified in groups. 176
Figure 61. Permutation tests for the validation of PLS-DA analysis of the samples detected in ESI- ionization mode classified in groups. 177

IRUDIEN ZERRENDA

Irudia 1. Europan 2018an diagnostikatutako 10 minbizi mota nagusien intzidentzia-tasa zenbatetsia. 184
Irudia 2. Melanoma intzidentzia AEB-etan, talde etniko ezberdinen arabera, 1975-2011 urteen artean 185
Irudia 3. Melanoma intzidentzia adina eta sexuaren arabera, AEB-tan 2007-2011 urteen artean 185
Irudia 4. 2018-ko larruazaleko melanomaren intzidentziaren banaketa zenbatetsia mundu osoan 186
Irudia 5. Fitzpatrick eskala 187
Irudia 6. Epidermisean dauden zelula mota nagusiak 190
Irudia 7. Clark eredua melanomaren garapena irudikatzeko 191
Irudia 8. Melanoma goiz detektatzeko ABCDE irizpideen adibideak 193
Irudia 9. Melanomaren garapen-etapa bakoitzaren deskribapen klinikoa 197
Irudia 10. Melanoma tratatzeko aukeren kronologia 2011tik 200
Irudia 11. Lipido mota ezberdinen egitura orokorra 205
Irudia 12. De novo sintesi-bideak PC, PE eta PS molekulak sortzeko. Epand et al.-etik hartuta. 209
Irudia 13. PI zikloa 211
Irudia 14. Glizerofosfolipidoen biosintesia. 214
Irudia 15. Esfingolipidoen egitura orokorraren irudikapen eskematikoa 215
Irudia 16. Esfingolipido-metabolismoaren bideen irudikapen eskematikoa 217
Irudia 17. Masa-espektrometro baten osagaien irudikapen eskematikoa 223
Irudia 18. Tandem MS/MS estrategiaren lan-fluxua eskematikoki adierazita 224
Irudia 19. Minbizi-zeluletako bidezidor metabolikoa erregulatzen duten seinaleztapen bideen irudikapen eskematikoa 226
Irudia 20. Minbizi zelulen lipido metabolismoan ematen diren aldaketen irudikapen eskematikoa 227
Irudia 21. Fosfolipasa ezberdinen hidrolizazio lekuak 230

TABLE INDEX

Table 1. Description of the criteria used to classify the staging of melanoma. 12
Table 2. Staging of melanoma based on TNM system 13
Table 3. Short description of the three most common fatty acid molecules 22
Table 4. Abbreviations of the lipid classes detected in this work 36
Table 5. Recovery rates (%) of the different standards performing the lipid extraction of a sample using Bligh & Dyer method and precipitation with isopropanol..... 38
Table 6. List of the reactants used for the experiments and the supplier 57
Table 7. Detailed description of the commercial cell lines used in this study 59
Table 8. Complete cell culture media composition for each cell line 60
Table 9. UHPLC-ESI-Q-TOF analysis condition. 68
Table 10. Percentage of acrylamide needed to resolve the proteins according to their molecular weight..... 74
Table 11. Composition of the running and stacking gels relying on the acrylamide percentage75
Table 12. Reagents used for casting the gels and their functions 75
Table 13. Loading buffer composition 75
Table 14. Primary antibodies used for western blot..... 77
Table 15. Secondary antibodies used for western blot..... 77
Table 16. Pathological classification of the cell lines studied by UHPLC-ESI-MS/MS methodology 83
Table 17. Validation of the PLS-DA models. 89
Table 18. Sum of the intensities obtained for all lipid species that make up each lipid subclass and comparison of the intensity variation of each subclass in the different study groups, related to control group (Melanocytes [M]=1) 90
Table 19. Sum of the intensities obtained for all the PC, PC(P/O), PE and PE(P/O) lipid species detected, and the ratios PC/PC(P/O), PE/PE(P/O), choline lipids/ethanolamine lipids, PC/PE, PC(P/O)/PE(P/O), LPC/LPE. The results of each study group are compared to the values obtained for skin melanocytes, that is considered the control group (Melanocytes [M]=1) 92
Table 20. Sum of the intensities obtained for all glycerolipid species (TG & DG) and comparison of the intensity variation of each class in the different study groups, related to control group (Melanocytes [M]=1)..... 95
Table 21. Sum of the intensities obtained for all lipid free fatty acid (FFA) species detected and comparison of the intensity variation of this class in the different study groups, related to control group (Melanocytes [M]=1)..... 96
Table 22. Sum of the intensities obtained for all lipid the species that make up each lipid subclass within the sphingolipids and comparison of the intensity variation of each subclass in the different study groups, related to control group (Melanocytes [M]=1) 97
Table 23. List of lipid potential biomarkers to discriminate between non-malignant and malignant cells..... 101
Table 24. List of lipid potential biomarkers to differentiate between nevus and primary melanoma cells 103
Table 25. List of lipid species that discriminate nevus melanocytes from metastatic melanoma cells..... 105
Table 26. List of lipid potential biomarkers of metastatic melanoma compared to primary melanoma cells 106
Table 27. Summary of the potential biomarkers 107

Table 28. List of lipids with significantly higher intensity in melanocytes (skin and nevus) or in melanoma cells (primary and metastatic) 120
Table 29. List of significant lipids with differential higher intensity in skin melanocytes or nevus melanocytes 124
Table 30. List of significant lipids with differential higher intensity in skin melanocytes or primary melanomas 124
Table 31. List of significant lipids with differential higher intensity in nevus melanocytes or primary melanomas. 126
Table 32. List of significant lipids with differential higher intensity in skin melanocytes or metastatic melanomas 127
Table 33. List of significant lipids with differential higher intensity in nevus melanocytes or metastatic melanoma 129
Table 34. List of significant lipids with differential higher intensity in primary or metastatic melanoma 130
Table 35. PC/PE ratio of the intensities obtained for the different PC and PE species that have the same acyl-chains, related to control group (skin melanocytes =1). 144
Table 36. PC/PS ratio of the intensities obtained for the different PC and PS molecules that share the same acyl-chains, related to control group (skin melanocytes=1) 145
Table 37. PE/PS ratio of the intensities obtained for the different PE and PS molecules that share the same acyl-chains, related to control group (skin melanocytes=1). 146
Table 38. SM/Cer ratio of the intensities obtained for the different SM and Cer molecules that share the same acyl-chains, related to control group (skin melanocytes=1). 147
Table 39. List of lipid molecules with significantly altered levels in the different statistical comparisons 151
Table 40. List of the detected lipid species by UHPLC-ESI-MS/MS approach, the media and standard deviation of their intensity, and the % of presence of each specie within its subclass 165

TAULEN ZERRENDA

Taula 1. Melanomaren estadifikazioa sailkatzeko erabiltzen diren irizpideen deskribapena .. 195
Taula 2.TNM sisteman oinarritutako melanomaren estadifikazioa. 196
Taula 1. Gantz-azido molekula arruntenen deskribapena. 206
Taula 4. Azilo albo-kate berdinak dituzten PC eta PE espezieen intentsitatearen PC/PE proportzioa, kontroleko taldeak lortutako emaitzekin erlazionatuta. 243
Taula 5. Azilo albo-kate berdinak dituzten PC eta PS espezieen intentsitatearen PC/PS proportzioa, kontroleko taldeak lortutako emaitzekin erlazionatuta 244
Taula 6. Azilo albo-kate berdinak dituzten PE eta PS espezieen intentsitatearen PE/PS proportzioa, kontroleko taldeak lortutako emaitzekin erlazionatuta 245
Taula 7. Azilo albo-kate berdina duten SM eta Cer espezieen intentsitatearen SM/Cer proportzioa, kontroleko taldeak lortutako emaitzekin erlazionatuta 246
Taula 8. Konparazio estatistikoetan euren mailak esanguratsuki aldatuta dituzten lipido espezieen zerrenda. 250

ABBREVIATIONS / LABURDURAK

	ENGLISH	EUSKARA
AA	Arachidonic acid	Azido arakidoniko
ACAT	Acyl-CoA:cholesterol acyltransferase	Azil-CoA:kolesterol aziltransferasa
ACC	Acetyl-CoA carboxylase	Azetil-CoA karboxilasa
ACDase	Acid ceramidase	Zeramidasa azido
ACLY	ATP citrate lyase	ATP zitrato liasa
ACS	Acyl-CoA synthetase	Azil-CoA sintetasa
ADAPS	Alkyl-DHAP synthase	Alkil-DHAP sintasa
ADP	Adenosine diphosphate	Adenosina difosfato
AGPS	Alkylglycerone phosphate synthase	Alkilglizerona fosfato sintasa
AIDS	Acquired immune deficiency syndrome	HIES-Hartutako immunoeskasiaren sindromea
AJCC	American Joint Committee on Cancer	Minbiziari buruzko Amerikako Komite Bateratua
ALM	Acral lentiginous melanoma	Melanoma akral lentiginosoa
ALT	Alanine aminotransferase	Alanina aminotransferasa
APAF-1	Apoptosis protease-activating factor-1	Apoptosiaren proteasa aktibatzen duen faktore-1
ATP	Adenosine triphosphate	Adenosina trifosfato
BCA	Bicinchoninic Acid Solution	Azido bizinkoniniko soluzioa
BCL-2	B-Cell lymphoma 2	B-zelulen linfoma 2
CCT	CTP:phosphocholine cytidylyltransferase	CTP:fosfokolina zitidiltransferasa
CDASE	Ceramidase	Zeramidasa
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Ziklina-menpeko kinasa inibitzailea 2A
CDP	Cytidine diphosphate	Zitidina difosfato
CDP-DG	Cytidine diphosphate diglyceride	Zitidina difosfato diglizerido
CE	Cholesterol ester	Kolesterol ester
CER	Ceramide	Zeramida
CERK	Ceramide kinase	Zeramida kinasa
CERS	Ceramide synthase	Zeramida sintasa
CERT	Ceramide transporter	Zeramida garraiatzaile
CK	Choline kinase	Kolina kinasa
CL	Cardiolipin	Kardiolipina
CMP	Cytidine monophosphate	Zitidina monofosfato
CDP	Cytidine diphosphate	Zitidina difosfato
CPT1	Carnitine palmitoyltransferase 1	Karnitina palmitoiltransferasa 1
CTLA-4	Cytotoxic T lymphocyte-associated protein 4	T linfozito zitotoxikoen proteina 4
CTP	Cytidine triphosphate	Zitidina trifosfato
DAG OR DG	Diglyceride or diacylglycerol	Diglizerido edo diazilglizerido
DAN	2,5-diaminonaphtalene	2,5-diaminonaftaleno
DCRIT	Maximum tolerable distance	Gehienezko distantzia onargarria

DGAT	Diacylglycerol acyltranferase	Diazilglizerol aziltransferasa
DHA	Docosahexaenoic acid	Azido dokosahexaenoiko
DHAP	Dihydroxyacetone phosphate	Dihidroxiazetona fosfato
DHAPAT	Dihydroxyacetonephosphate acyltransferase	Dihidroxiazetonafosfato aziltransferasa
DMODX	Distance to the model in X-space	X-planoan egindako eredurako distantzia
DMSO	Dimethyl Sulfoxide	Dimetil sulfoxido
DNA	Deoxyribonucleic acid	Azido desoxirribonukleiko
DTT	Dithiothreitol	Ditiotreitol
ECL	Enhanced ChemiLuminescence	Areagotutako kimioluminiszentzia
ECT	CTP:phosphoethanolamine cytidylyltransferase	CTP:fosfoetanolamina zitidiltransferasa
EGF	Epidermal growth factor	Hazkuntza faktore epidermiko
EGFR	Epidermal growth factor receptor	Hazkuntza faktore epidermikoaren hartzaile
EK	Ethanolamine kinase	Etanolamina kinasa
ELOVLS	Elongases of very long fatty acids protein	Kate oso luzeko gantz azidoen elongasak
EMA	European Medicines Agency	Europako Medikamentuen Agentzia
EPA	Eicosapentaenoic acid	Azido eikosapentaenoiko
EPT	Ethanolamine phosphotransferase	Etanolamina fosfotransferasa
ER	Endoplasmic reticulum	Erretikulu endoplasmiko
ERK	Extracellular signal-regulated kinase	Zelula-kanpoko seinalez erregulaturiko kinasa
ESI	Electrospray ionization	Elektroesprai bidezko ionizazioa
H_2O	Water	Ur
FA	Fatty acid	Gantz azido
FABP7	Fatty acid-binding protein 7	Gantz azidoetara lotzen den proteina 7
FADS	Fatty acid desaturase	Gantz azido desaturasa
FAMMM	Familial Atypical Multiple Moles and Melanoma	Orezta atipiko aniztun melanoma familiarra
FAO	Fatty acid oxidation	Gantz azidoen oxidazio
FAS	Fatty acid synthase	Gantz azido sintasa
FBS	Fetal Bovine Serum	Behi serum fetala
FDA	Food and Drug Administration	Elikagai eta Farmakoen Administrazioa
FFA	Free Fatty Acid	Gantz azido askea
G3P	Glycerol-3-phosphate	Glizerol-3-fosfato
GALCER	Galactosylceramide	Galaktosilzeramida
GCS	Glucosylceramide synthase	Glukosilzeramida sintasa
GEF	Guanine nucleotide exchange factor	Guanina nukleotidoa trukatzeko faktore
GL	Glycerolipids	Glizerolipido
GLCCER	Glucosylceramide	Glukosilzeramida
GPL	Glycerophospholipid	Glizerofosfolipido
GM-CSF	Granulocyte-macrophage colony-stimulating factor	Granulozito-makrofagoen koloniak estimulatzeko faktore
GP-100	Glycoprotein 100	Glikoproteina 100

GPAT	Glycerol-3-phosphate acyltransferase	Glizerol-3-fosfato aziltransferasa
HBSS	Hank's Balanced Salt Solution	Hank soluzio gazi orekatua
HEXCER	Hexosylceramide	Hexosilzeramida
HIF-1	Hypoxia Inducible Factor–1	Hipoxia-eragile faktorea 1
HMB45	Human Melanoma Black-45	Giza melanoma beltza 45
HMGB1	High mobility group box 1	Mugikortasun handiko box 1 talde
HMG-COA	Hydroxymethylglutaryl CoenzimeA	Hidroximetilglutaril KoentzimaA
HMGR	HMG-CoA reductase	HMG-CoA erreduktasa
HMGS	Human Melanocyte Growth Supplement	Giza melanozitoen hazkuntzarako osagarria
HRP	Horseradish peroxidase	Errefau peroxidasa
HSV1	Herpes simplex virus	Herpes simplex birusa
IFN-A	Interferon-alpha	alpha-interferona
IL-2	Interleukin-2	2 Interleukina
IL-8	Interleukin-8	8 Interleukina
INK4A	Cell-cycle inhibitor of kinase 4A	Zelula-zikloaren inhibitzailea den kinasa 4A
JAK3	Janus kinase 3	Janus kinasa 3
LCL	Lysocardiolipin	Lisokardiolipina
LD	Lipid droplets	Gantz tantak
LDH	Lactate dehydrogenase	Laktato deshidrogenasa
LMM	Lentigo maligna melanoma	Lentigo maligna melanoma
LPA	Lysophosphatidic acid	Azido lisofosfatidiko
LPAAT	LysoPA acyltransferase	LisoPA aziltransferasa
LPC	Lysophosphatidylcholine	Lisofosfatidilkolina
LPCAT	LPC acyltransferase	LPC aziltransferasa
LPCLAT	LCL acyltransferase	LCL aziltransferasa
LPE	Lysophosphatidylethanolamine	Lisofosfatidiletanolamina
LPEAT	LPE acyltransferase	LPE aziltransferasa
LPG	Lysophosphatidylglycerol	Lisofosfatidilglizerol
LPGAT	LPG acyltransferase	LPG aziltransferasa
LPI	Lysophosphatidylinositol	Lisofosfatidilinositol
LPIAT	LPI acyltransferase	LPI aziltransferasa
LPLAT	Acyl-CoA:lysophospholipid acyltransferases	Azil-CoA:lisofosfolipidoen aziltransferasa
LPS	Lysophosphatidylserine	Lisofosfatidilserina
M	Skin melanocytes	Larruazaleko melanozitoak
M/Z	mass-to-charge ratio	masa-karga ratioa
MAGL	Monoacylglycerol lipase	Monoazilglizerol lipasa
MALDI	Matrix-assisted laser desorption and ionization	Laser bidezko desortzioa eta ionizazioa matrize bidez lagunduta
MAPK	Mitogen-activated protein kinase	Mitogenoz aktibatutako proteina kinasa
MBT	2-mercaptobenzothiazole	2-merkaptobezotiazol
MCT	Monocarboxylate transporter	Monokarboxilato garraiatzaile
MG	Monoglyceride	Monoglizerido
MM	Metastatic melanoma	Melanoma metastatiko

MMGL	Lymph-node metastatic melanoma	Gongoil linfatikoetako melanoma metastatiko
MMNS	Subcutaneous metastatic melanoma	Larruazalpeko melanoma metastatiko
MP	Primary melanoma	Melanoma primario
MRNA	Messenger RNA	RNA mezulari
MS	Mass spectrometry	Masa espektrometria
MS/MS	Tandem mass spectrometry	Tandem masa-espektrometria
MTOR	Mammalian target of rapamycin	Rapamizinaren itua ugaztunetan
MUFA	Monounsaturated fatty acids	Gantz azido monoinsaturatua
N	Nevus melanocytes	Nevus melanozitoak
NADPH	Nicotinamide adenine dinucleotide phosphate	Nikotinamida adenina dinukleotido fosfato
NER	Nucleotide excision repair	Nukleotidoen erauzketa bidezko konponketa
NM	Nodular melanoma	Melanoma nodular
NMR	Nuclear magnetic resonance	Erresonantzia magnetiko nuklear
OPLS-DA	Orthogonal partial least squares discriminant analysis	Minimo karratu partzial ortogonalak bereizteko analisia
OXPHOS	Oxidative phosphorylation	Fosforilazio oxidatibo
PA	Phosphatidic acid	Azido fosforiko
PAF	Paraformaldehyde	Paraformaldehido
PAGE	Polyacrilamide gel electrophoresis	Poliakrilamidazko gelen bitarteko elektroforesia
PAP	Phosphatidic acid phosphatase	Azido fosfatidikoaren fosfatasa
PBS	Phosphate saline buffer	Fosfato gatz soluzio indargetzailea
PC	Phosphatidylcholine	Fosfatidilkolina
PC(P/O)	Phosphatidylcholine ether	Fosfatidilkolina eter
PC8	Short side-chain phosphatidylcholine [PC(8:0/8:0)]	Kate-laburreko fosfatidilkolina [PC(8:0/8:0)]
PCA	Principal Components Analysis	Osagai nagusien analisia
PD-1	Programmed cell death protein 1	Programatutako zelula heriotzaren proteina 1
PDAT	Phospholipid:diacylglycerol acyltransferase	Fosfolipido:diazilglizerol aziltransferasa
PDGF	Platelet-derived growth factor	Plaketetatik eratorritako hazkuntza faktorea
PDGFR	Platelet-derived growth factor receptor	Plaketetatik eratorritako hazkuntza faktorearen hartzailea
PD-L1	Programmed cell death ligand 1	Programatutako zelula heriotzaren proteina 1-aren hartzailea
PE	Phosphatidylethanolamine	Fosfatidiletanolamina
PE(P/O)	Phosphatidylethanolamine ether	Fosfatidiletanolamina eter
PEMT	Phosphatidylethanolamine methyltransferase	Fosfatidiletanolamina metiltransferasa
PG	Phosphatidylglycerol	Fosfatidilglizerol
PH	Pleckstrin homology domain	Pleckstrina homologia duen domeinua
PI	Phosphatidylinositol	Fosfatidilinositol
PI3K	Phosphatitylinositol 3-kinase	Fosfatidilinositol 3-kinasa

PIP	Phosphatidylinositol phosphate	Fosfatidilinositol fosfato
PIP_2	Phosphatidylinositol 4,5-biphosphate	Fosfatidilinositol 4,5-bifosfato
PIP_3	Phosphatidylinositol triphosphate	Fosfatidilinositol trifosfatoa
PKC	Protein kinase C	Proteina kinasa C
PLA_2	Phospholipase A_2	A_2 Fosfolipasa
PLB	Phospholipase B	B Fosfolipasa
PLC	Phospholipase C	C Fosfolipasa
PLD1	Phospholipase D1	D1 Fosfolipasa
PLD2	Phospholipase D2	D2 fosfolipasa
PLS-DA	Partial least squares discriminant analysis	Minimo karratu partzialak bereizteko analisia
PMEL	Premelanosome protein	Premelanosoma proteina
PPI	Pyrophosphate	Pirofosfato
PPIN	Phosphoinositides	Fosfoinositidoak
PPP	Pentose phosphate pathway	Pentosa fosfatoen bidea
PRB	Retinoblastoma protein	Erretinoblastoma proteina
PS	Phosphatidylserine	Fosfatidilserina
PSD	Phosphatidylserine decarboxylase	Fosfatidilserina descarboxilasa
PSS	Phosphatidylserine synthase	Fosfatidilserina sintasa
PSS1	Phosphatidylserine synthase 1	Fosfatidilserina sintasa 1
PSS2	Phosphatidylserine synthase 2	Fosfatidilserina sintasa 2
PTEN	Phosphatase and tensin homologue	Fosfatasa eta tensinaren homologoa
PUFA	Polyunsaturated fatty acid	Gantz azido poliinsaturatua
PX	Phox homology domain	Phox homologia duen domeinua
QC	Quality Control	Kalitate-Kontrola
Q-TOF	Quadrupole-time-of-flight	Kuadrupolo-hegaldi-denbora
RGP	Radial growth phase	Hazkuntza erradialeko fasea
RNA	Ribonucleic acid	Azido erribonukleiko
RTK	Receptor tyrosine kinase	Tirosina kinasa hartzaile
S1P	Sphingosine 1-phosphate	Esfingosina 1-fosfato
SCD	Stearoyl-CoA desaturases	Estearoil-CoA desaturasa
SDS	Sodium Dodecyl Sulfate	Sodio dodezil fosfato
SDS-PAGE	SDS-polyacrylamide gel electrophoresis	SDS-poliakrilamida gelen bidezko elektroforesia
siRNA	Small interfering RNA	Interferentziako RNA laburra
SK	Sphingosine kinase	Esfingosina kinasa
SM	Sphingomyelin	Esfingomielina
SMASES	Sphingomyelinases	Esfingomielinasa
SMS	Sphingomyelin synthase	Esfingomielina sintasa
SMS1	Sphingomyelin synthase 1	Esfingomielina sintasa 1
SPL	Sphingolipids	Esfingolipidoak
SPT	Serine palmitoyltransferase	Serina palmitoiltransferasa
SREBP	Sterol regulatory element-binding proteins	Esterola erregulatzen duen elementuarekin lotzeko proteinak

SSM	Superficial spreading melanoma	Azalerako hedadura duen melanoma
TBS-T	TBS-Tween 20	TBS-Tween 20
TCA	Tricarboxylic acid cycle	Azido trikarboxilikoen zikloa
TCR	T-cell receptor	T zelulen hartzaile
TG	Triglyceride	Triglizerido
TIC	Total iont current	Guztizko ioien korrontea
TILS	Tumor infiltrating lymphocytes	Tumorean infiltraturiko linfozitoak
TLC	Thin layer chromatography	Geruza finean egindako kromatografia
TVEC	Talimogene laherparepvec	Talimogene laherparepvec
UHPLC	Ultra-high pressure liquid chromatography	Presio ultra handiko kromatografia likidoa
UICC	Union for International Cancer Control	Minbiziaren kontrolerako nazioarteko batasuna
UV	UltraViolet light	Erradiazio ultramore
VEGF	Vascular Endothelial Growth Factor	Endotelio baskularraren hazkuntza faktore
VGP	Vertical growth phase	Hazkuntza bertikaleko fasea
VIP	Variable Importance in Projection	Aldagaien inportantzia proiekzioan

INTRODUCTION

1. Melanoma

Melanoma is the cancer that arises upon the malignant transformation of the melanocytes that acquire the ability to grow and proliferate uncontrollably. Melanocytes generate melanin, which spreads to neighboring cells and protects them from UV radiation. Although the main majority of melanomas occur in the skin, there are also non-skin melanomas, such as, uveal and mucosal melanomas, among others. Despite representing only 4% of all skin tumors diagnosed, skin melanoma is the most lethal form, since it is responsible for 80% of the deaths caused by these cancers. Moreover, every hour a person dies in the U.S.A. due to melanoma[1].

1.1. Epidemiology

1.1.1. Incidence

The incidence of melanoma has steadily increased during the last decades throughout the world. For instance, in the United States in 1930, the probability of suffering from melanoma was 1 in 1,500; however, in 2011 that risk was 1 in 52[2]. Concretely in Spain, it is predicted that there will be 150,000 new cases in 2019[3]. Furthermore, it is estimated that it was the seventh type of cancer with the highest incidence in Europe in 2018, affecting 11.2 people per 100,000 **(Fig. 1)**.

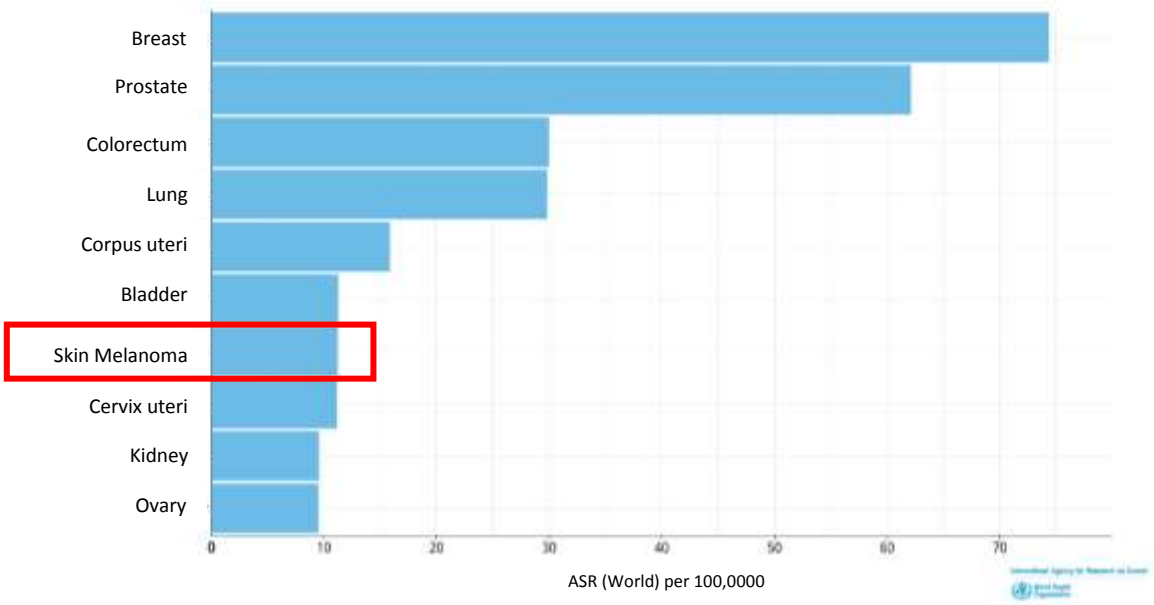


Figure 1. Estimated incidence rates of the diagnosed top 10 cancer types in Europe in 2018. Both sexes and all ages are considered[3].

The incidence varies according to ethnicity, sex, age and the geographical region studied. Concretely, the incidence of melanoma is reasonably higher among fairly-skinned Caucasians. As it is known, UV radiation is the main risk factor for melanoma, so the melanin of the darker-pigmented individuals forms a barrier that protects cells from the carcinogenic effects of sunlight. Although melanoma is disproportionately related to Caucasians, the overall five-year survival rate is lower for African Americans, since the diagnosis is usually made earlier in the individuals with milder pigmentation. Furthermore, the tumors arise in different areas depending on the ethnicity; in Caucasians, it tends to appear in sun-exposed areas, while in dark-

skinned people it usually develops in non-sun-exposed areas such as mucous membranes, nail beds, and the palms of hands and soles of the feet. Thus, making it more difficult to detect the tumors[4].

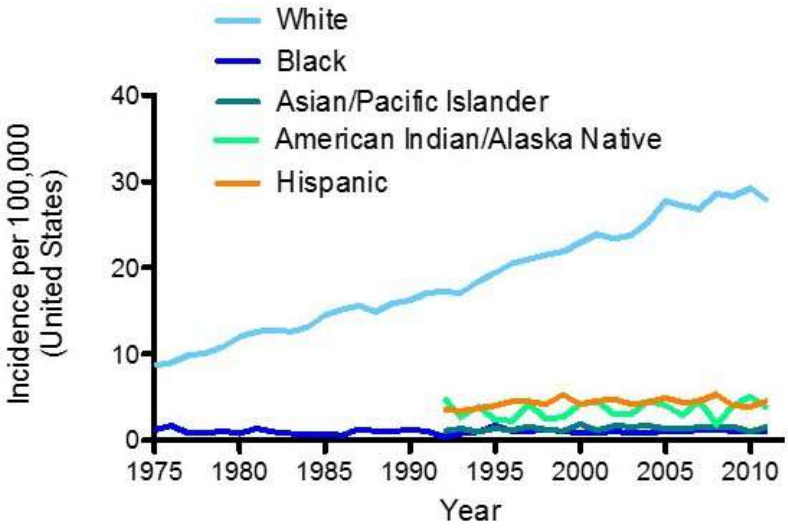


Figure 2. Incidence of melanoma in the U.S. according to the different ethnic groups, between the years 1975-2011[4].

Melanoma affects each sex differently. Besides, the incidence rates also depend on the age of the patient. Melanoma is more common among adolescent and young women, than in men. However, at the age of 40, the trends are reversed and men are more likely to suffer from melanoma. Altogether, the incidence rates are higher in men. Regardless of the increase in the incidence in both sexes, the rates have spread massively among women under 40 years, probably due to the popularization of the use of tanning beds and having a tanned complexion among women[5].

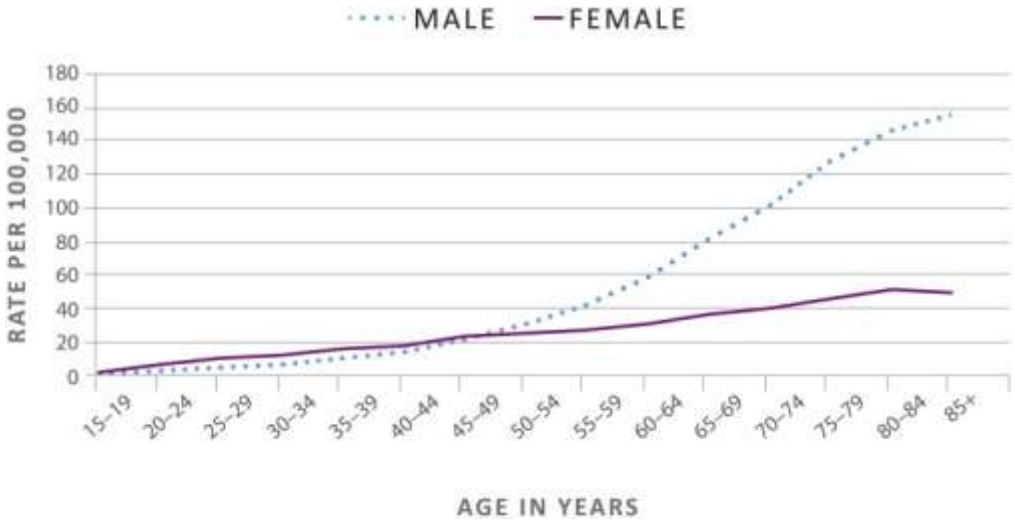


Figure 3. Melanoma incidence by age and sex, during 2007-2011 in the United States[5].

Even among individuals of the same ethnic group, the incidence of melanoma varies according to geographical location. Taking into account lightly-pigmented individuals, countries located close to the equator have higher rates of melanoma, since they are exposed to higher UV intensities. Notably, the countries with the highest rates of melanoma are New Zealand and Australia **(Fig. 4)**, as they have high rates of lightly-pigmented individuals and are found in lower latitudes. However, within Europe, an inverse latitude gradient has been perceived. In fact, central and northern countries such as Switzerland, the Netherlands, Denmark, Norway and Sweden present a three-to-six times higher risk of melanoma than in southern countries. This could be partly due to the lighter skin phototype of the population these countries and the sun exposure pattern.

While people in northern countries have occasional sun exposure and sunburn easily, individuals in southern countries have a cumulative sun exposure. Significantly, a north-south incidence gradient has been observed within the population of Australia and the Scandinavian countries, where the people living in closer to the equator present a greater risk of melanoma[6].

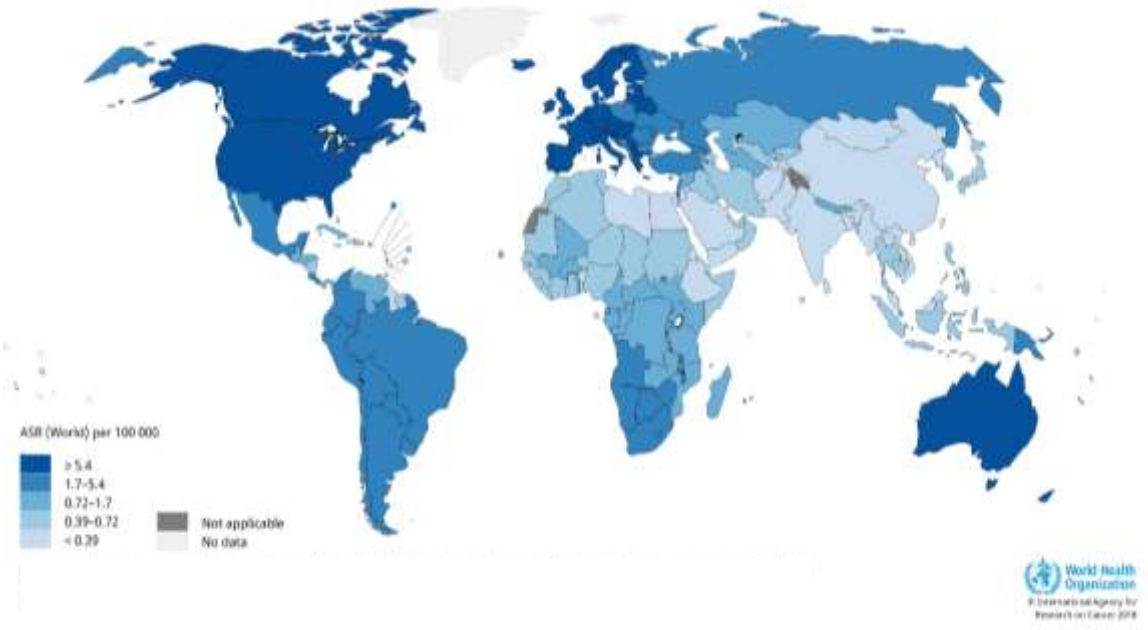


Figure 4. Estimated worldwide skin melanoma incidence distribution for 2018[3]. Dark blue represents 5.4 melanoma cases per 100,000 inhabitants, while light blue represents less than 0.39 melanoma cases per 100,000 inhabitants.

1.1.2. Mortality

Similarly to incidence, melanoma mortality rates are also related to sex, age, ethnicity and geographical location. However, mortality rates have not followed the same trends as incidence. In fact, in the last 20 years they have stabilized, probably due to the advances in awareness, early diagnosis and medical and surgical care[4]. Equally to incidence, melanoma mortality is higher in the low latitude regions near the equator. According to sex and age, men have higher mortality rates than women worldwide, and the peak of mortality is beyond the seventh decade of life. Within the ethnic groups in the United States, mortality is greater among Caucasians than among African-Americans. However, even if Caucasians are more prone to suffer melanoma,

this is usually detected at an earlier stage than in dark pigmented ethnic groups. Thus, 5-year survival is lower for African-Americans than for Caucasians. In fact, the 5-year survival rate has risen among Caucasians over the past decade, while it has declined for African-Americans. However, the socioeconomic status has been blamed for this trend[5].

1.2. Etiology

Although the etiology of melanoma is still unknown, some risk factors have been identified to favor the appearance and development of this cancer. These factors can be divided in intrinsic factors that are inherent to the patient and extrinsic or environmental factors.

1.2.1. Intrinsic factors

As mentioned above, the **age** and **sex** of the patient are important factors in determining the probability of developing melanoma. In this regard, there is a greater overall risk of melanoma with advanced age for both women and men, with the average age of diagnosis being 60 years. In general, there is a higher risk in males, with a ratio of 2 men: 1 women at age 80; conversely, as seen in F**igure 3**, the rate is greater for women under 40 years of age in the U.S.[2].

The amount of melanin in the skin of an individual defines its **skin phototype**, which determines the patient's susceptibility to developing melanoma. In 1975, Thomas B. Fitzpatrick established the Fitzpatrick scale that numerically categorizes an individual's vulnerability to UV, and its risk to consequently develop melanoma based on its tendency to tan and burn, and its basal levels of pigment. Individuals with pale skin, blond or red hair, freckles, high tendency to burn and inability to tan have been associated with a low Fitzpatrick score and are more likely to develop melanoma **(Fig. 5)**. In contrast, people with a higher Fitzpatrick score have darker skin, do not burn, tan easily, and this is associated with a relative lower risk of developing melanoma[2,4].

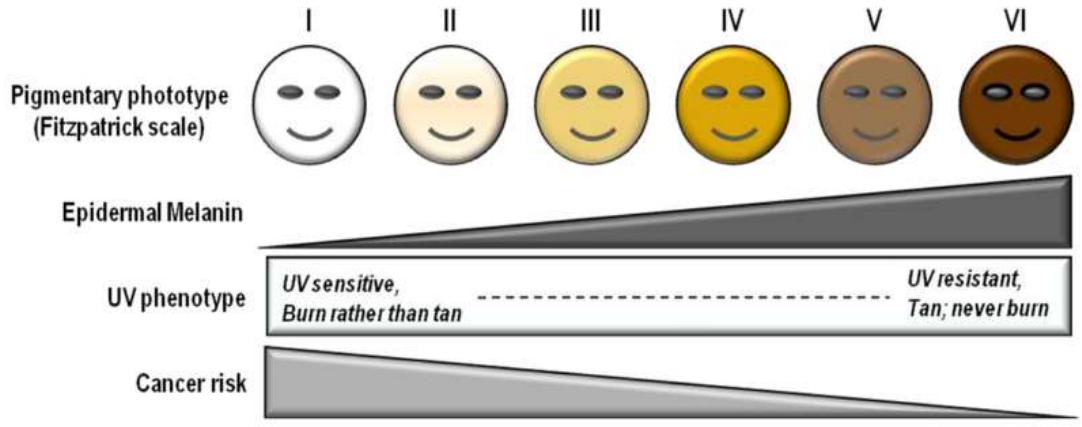


Figure 5. Fitzpatrick scale. Numerical classification to determine the susceptibility of an individual to UV radiation and melanoma development. Taken from[7].

Only 5-10% of melanoma cases have a **genetic component**, but the **family history** of melanoma has been related to an increased risk of developing this disease. Notably, if a primary relative has suffered melanoma, the risk is 1.7 higher, and if one parent has multiple melanoma, the risk is 61.78 greater[2,5]. Although it is still unknown if this increased risk is due to an inherited genetic predisposition or shared environmental factors, some genetic disorders have been linked to an

increased risk of developing melanoma. The most studied disorder is Xeroderma pigmentosum[2,4,7]. This is an autosomal recessive condition that increases the risk in a 1000-fold. Patients with this syndrome have a defective nucleotide excision repair (NER) that is the responsible for correcting DNA lesions. Thus, the cells are unable to repair the DNA damage generated by UV radiation, and individuals develop UV hypersensitivity and 65% of the tumors originate in sun-exposed anatomical areas. Another alteration is Familial Atypical Multiple Moles and Melanoma (FAMMM) syndrome. This is described as having two or more direct relatives with a history of both melanoma and dysplastic nevi. In particular, at age 50, an individual diagnosed with this syndrome has a 49% greater risk of suffering from melanoma, and 82% at 72 years. This disorder is caused by mutations in the CDKN2A gene, which encodes p16 and p14 tumor suppressor proteins[2,4,5,7]. Furthermore, in 90% of melanoma cases there is an enhanced expression of Bcl-2 protein, which is an apoptosis inhibitor. The mTOR pathway, a pivotal regulator of the cell cycle, is activated in 67-77% of cases. The gain-of-function mutation of MAPK/ERK pathway generates the constant activation of BRAF kinase in 60-80% of the melanomas, favoring the proliferation of the melanocytes[8].

In addition, previous **personal history** of any type of skin cancer increases the chance of having melanoma. Unsurprisingly, people with melanoma have a 8% risk of having a second one[4,7]. Besides, it has been reported that other personal medical conditions are also related to an increased likelihood of developing melanoma. As it is known, the immune system plays a key role in protecting the body against cancer. Therefore, people with AIDS have an elevated chance of developing melanoma. Similarly, people who have received an organ transplant are 6% more likely to have melanoma if the transplant was during adulthood, and a 14% chance in children's transplants. In addition, a link with breast cancer has been detected. People who have had breast cancer and are carriers of the BRCA2 mutation have a relative risk of 2.58 to develop melanoma. Among patients with a history of lymphocytic leukemia or non-Hodgkin's lymphoma, there is a greater chance of having melanoma, since this is one of the most probable secondary cancer in both conditions[2].

The presence of a large number of **nevi** has been connected to a higher chance of having melanoma. Most nevi and melanoma share the BRAF mutation, which is able to create nevus, but additional mutations are needed for melanoma development. The malignant transformation of typical moles is rare, whereas the presence of dysplastic nevi increases the risk. In fact, a dysplastic nevus increases the risk by two, while 16 to 40 typical moles increase the risk by 1.47. Significantly, no association has been detected between congenital nevi and melanoma development[2,4,7].

1.2.2. Extrinsic factor

UV radiation has been clearly related to the appearance of melanoma. In fact, it is considered the most important carcinogen for this condition, since it is responsible for 80% of the cases[4]. UV radiation generates DNA damage and cell injury by inducing mutations. Defective DNA repair machinery cannot replace the damaged DNA sequences and melanoma arises. The exposure pattern also plays a role. Recreational or intermittent exposure, especially in light-skinned vacationers, results in a 65% higher risk of melanoma and nevi. However, it has been shown that the use of sunscreens reduces melanoma incidence rate, especially that of invasive melanomas[2]. During the last decades, the use of tanning beds has become very popular, however, this social activity is strongly linked to melanoma. Notably, several meta-analyzes suggest that the frequent use of tanning beds triples or quadruples the risk of developing melanoma, and increases this risk to 75% if the first artificial UV exposure was performed before the age of 35[7].

Since countries located closer to equator are exposed to higher UV intensity, this results in higher melanoma rates, highlighting the importance of **geographic location**. As mentioned before, Australia and New Zealand, which are close to equator and have a fair-skinned population, have the greatest rates of melanoma. Despite being an important feature, geographical location is not determinant. For instance, in spite of being close to equator, Central America has low melanoma rates, since most people are dark-skinned.

A link has been established between **exposure to heavy metals, various chemicals,** and the development of melanoma, since these compounds generate mutations in the DNA of the melanocytes. Occupational exposure to ionizing radiation, heavy metals, polycyclic hydrocarbons (petroleum, printing chemicals, and electronic products), pesticides and polyvinyl chloride increases the risk of melanoma. However, the mechanism for this is still under study[9].

1.3. Histopathology

Due to the neural crest-origin of melanocytes, melanoma can occur in the uveal tract and on the mucosal surface, for example, but in 95% of cases, it arises in its cutaneous form.

1.3.1. Skin

Skin is the largest organ of the body, covering an area of 2 m^2. It acts as a protective shield against heat, light, pathogens and injuries. In addition, it also helps to regulate body temperature, vitamin D synthesis, cutaneous sensitivity (touch, heat and cold) and it prevents water loss.

According to its structure, it is divided into two different portions: epidermis and dermis[10]. Besides, underneath the dermis, but without belonging to the skin, is the hypodermis, which can also be called subcutaneous tissue. It is composed mainly of loose connective tissue and fat accumulations. The cellular content of the hypodermis is made up mainly of adipocytes, fibroblast and macrophages. Its principal function is to store fat and help maintain body temperature.

The **epidermis** is the outermost layer of the skin, and acts as a barrier to the internal structures of the body, regulates the hydration of the skin and provides color to the skin. It is composed of a thin stratified squamous epithelium that lies on a basement layer that separates it from the dermis. Among all the cells found in this structure, the main ones are keratinocytes, melanocytes, Langerhans cells and Merkel cells **(Fig. 7)**. A 90% of these cells are keratinocytes, which produce keratin, a resistant and fibrous protein that protects the skin. In addition, the cells responsible for producing melanin and determining skin color are melanocytes, which represent 8% of the cells of the epidermis. Also, there are Langerhans cells, which participate in the reinforcement of the immune response against the microorganisms present in the skin, and Merkel cells, which make synaptic contact with sensitive neurons and act as mechanoreceptors for tactile sensation.

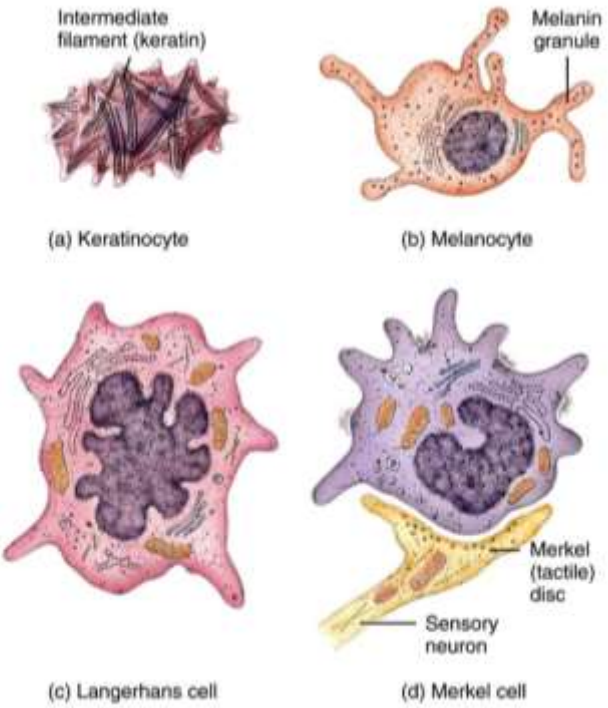


Figure 6. The principal cell types present in the epidermis: (a) keratinocytes, (b) melanocytes, (c) Langerhans cells and (d) Merkel cells[10].

Beneath the epidermis is the **dermis**. This is a thick layer of connective tissue that contains blood vessels, nerves, glands and hair follicles, and is tightly connected to the epidermis by the basement layer. Therefore, it acts as support, nourishment and waste removal for the cells of the epidermis and dermis.

Since **melanocytes** are responsible for the development of melanoma, we have focused our attention on this type of cells. They are derived from the embryonic ectoderm. Hence, after the closure of the neural tube, the melanoblasts, precursors of the melanocytes, migrate from the neural crest to different parts of the body, such as skin, uveal tract, mucosal surfaces, meninges, inner ear and heart. This is the reason why melanoma could arise in any of these locations, although it develops mainly on the skin.

The density of the melanocytes varies depending on the anatomic region of the body (higher in the breast aura and the genital region). However, it remains fairly constant between individuals of different ethnicities. Therefore, the color of the skin is determined by the quantity of melanin produced instead of the number of melanocytes.

Under physiological conditions, melanocytes are found mainly on the basal layer of the epidermis, attached to up to 36 keratinocytes through their dendritic projections, forming the epidermal melanin unit. Melanocytes have a low replication capacity, light cytoplasm and do not present desmosomes. Besides, their main role is to fabricate melanin, the photoprotective pigment that acts as a barrier for the skin, since it blocks the harmful effects that UV radiation could cause on cells, such as oxidative stress and DNA mutagenesis. Inside the melanocytes there are the melanosomes organelles, where melanin is manufactured and stored. Then, these vesicles are distributed to the surrounding keratinocytes of the epidermal melanin unit via the dendritic projections of the melanocytes.

1.3.2. Melanoma development

As mentioned above, skin melanoma arises after the malignant transformation of the melanocytes present in the epidermis of the skin. These cells undergo different molecular alterations, among which there are mutations in genes that control cell cycle regulation, cell differentiation, cell adhesion, cell signaling and apoptosis[11].

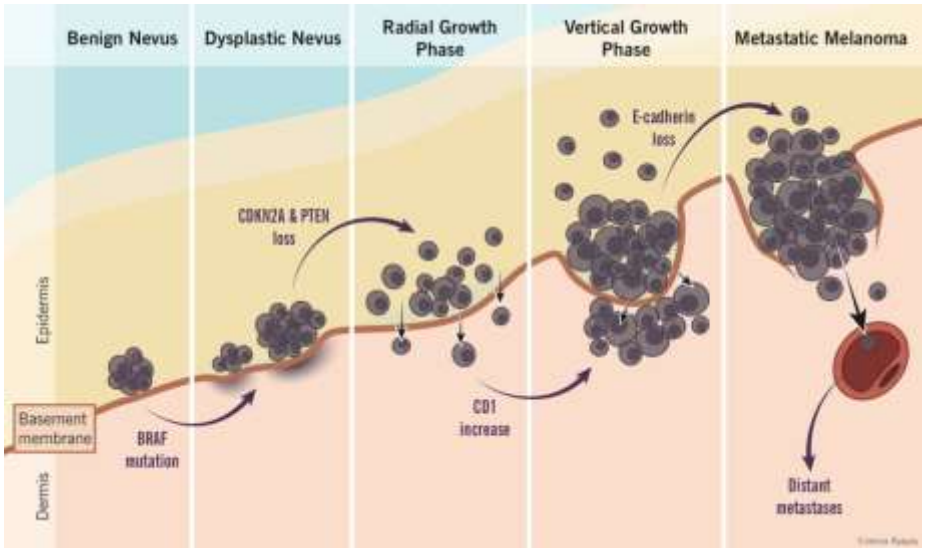


Figure 7. Representation of the Clark model for melanoma development [12].

Different models have been proposed to explain melanoma development. One of the most popular is the Clark model, which, as represented in the **Figure 7**, explains the melanoma development in different steps: benign nevi, dysplastic nevi, primary melanoma in the radial growth phase (RGP), primary melanoma in the vertical growth phase (VGP) and metastatic melanoma. Although this model of progression is explained as a linear stepwise transformation, many melanoma tumors may not follow it in an orderly fashion. For example, RGP or VGP melanomas can arise from pre-existing nevi lesions or *de novo* from normal melanocytes. Also, either RGP or VGP tumors can progress directly to metastatic tumors[12].

The first event in Clark's progression model is the formation of benign nevi, since the epidermal melanocytes undergo some molecular changes that alter their growth control. However, the proliferation of nevus melanocytes is limited; the cells enter into senescence induced by oncogenes and advance scarcely towards melanoma. Clinically, they can be described as brown moles with regular surface and borders. Some of the identified molecular changes are the aberrant activation of the MAPK signaling pathway, which results in an increased proliferation of melanocytes. This constitutive activation of the ERK-MAPK pathway occurs due to mutations in N-RAS, observed in 15% of melanomas, or BRAF, associated with 50% of melanoma cases. The frequency of BRAF mutations is similar to that observed in melanomas. Therefore, additional alterations must occur so that the melanocytes become malignant. Some authors suggest that the BRAF mutation induces cellular senescence because it increases the expression of the cell-cycle inhibitor of kinase 4A (INK4A). This protein causes cell-cycle arrest and the stimulation of growth produced by BRAF mutation is limited[12].

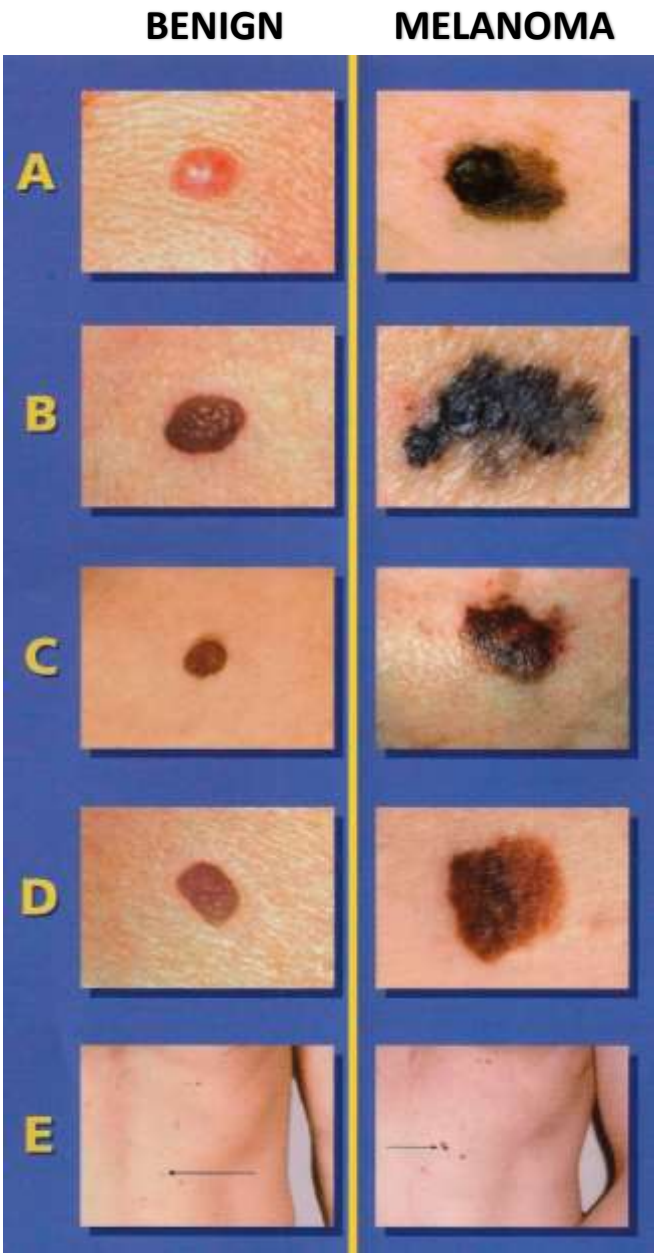
This benign tumor can progress to dysplastic nevus, which are considered precancerous lesions between benign nevus and melanoma. They can arise from pre-existing benign nevus or as new lesions. Despite being considered risk factors and having the ability to be precursors of melanoma, the majority of the dysplastic nevus remain stable over time and do not progress to melanoma. In fact, only 20% of melanoma cases develop from these precancerous lesions. Several studies have concluded that the risk of a nevus becoming melanoma is 1 in 200,000 for patients under 40 and 1 in 33,000 for men over 60[13]. Clinically, these moles are larger than 5 mm, with irregular borders and variegated color, and histologically, the cells show cytological atypia. At the molecular level, they undergo alterations that modify cell growth, DNA repair machinery and susceptibility to cell death[12].

Eventually, the cells can acquire the ability to grow limitlessly and form the malignant tumor. Moreover, melanomas have two distinct growth phases that will determine their clinical outcome. The first phase RGP, is where the tumor grows laterally along the epidermis and do not generate metastasis. This could last for years and the tumor can be surgically removed with a recovery rate close to 100%. The second phase is VGP, in which the tumor acquires the ability to grow deeply and invades the dermis and hypodermis, resulting in metastasis.

Melanoma is one of the most aggressive tumor types, and survival rates drop dramatically when diagnosed in the metastatic phase. To form a metastasis, the cells lose cell-cell adhesions, separate from the primary tumor, and invade the surrounding stroma. Melanoma cells use different migration mechanisms to spread: intravascular dissemination through lymphatic or blood vessels, or extravascular migration called angiotropism. The latter is defined as the dissemination of the tumor without entering the vasculature, since the cells mimic the pericytes of the vessels and migrate along the abluminal vascular surfaces without intravasation. Melanoma cells share this migratory mechanism with neural crest cells[14,15]. The tumor can metastatize loco-regionally or to distant sites. Commonly, it metastatizes regionally to nearby skin (satellite or in-transit metastasis), lymph nodes and subcutaneous tissue. Besides, the most common distant metastases are to skin, lung, brain, liver, bone and intestine[16].

1.3.3. Melanoma diagnosis and staging

Survival rates are significantly higher with early detection of the tumor. However, if the lesion is not surgically removed in time and continues to grow, it becomes in one of the deadliest cancers. Therefore, it is strikingly important to have resources to help with early detection. Melanoma tumor can be identified if it meets the ABCDE criteria[17]:



Asymmetry: the mole has two different halves. Early lesions usually grow at an uneven rate, so the mole becomes asymmetrical.

Border irregularity: the borders of the mole are not well defined and irregular.

Color variation: inside the same mole, there are color variations. It may have different brown or black tones, or even white, blue and red.

Diameter: melanomas usually have a diameter greater than 6 mm.

Evolution: when a mole is different in appearance from the other moles of the patient, or, if the same mole changed (size, color, shape, etc.) over a period, is suspected to be melanoma.

Figure 8. Examples of the ABCDE criteria for the early detection of melanoma[20].

Unfortunately, the ABCDE criteria allows for the correct diagnosis of only 60-65% of cases, so additional criteria are needed to aid in the diagnosis and prognosis of melanoma. For this, the Clark scale and the Breslow scale are employed together.

The Clark scale determines invasion level of the tumor and the skin layers affected. Wallace H. Clark developed this staging system in 1966 and 5 different levels are recognized:

- I: melanoma cells are only confined to the epidermis. It is also called melanoma *in situ*.
- II: the cells invade the papillary dermis, right under the epidermis.
- III: melanoma cells invade the junction of the papillary and reticular dermis.
- IV: the melanoma invades the reticular or deep dermis.
- V: the tumor grows to the subcutaneous fat beneath the dermis.

However, the Clark scale is rarely used nowadays because it has been shown to have a lower predictive value, be more subjective and less reproducible than the Breslow scale. Furthermore, it is often difficult to differentiate between Clark's level II and III, and cannot be used for melanomas on the soles and palms. Thus, the Clark scale has been relegated to cases where the Breslow depth is less than 1 mm[18].

The Breslow scale was reported by Alexander Breslow in 1970 and determines how deeply the tumor has grown. The thickness of the excised tumor is measured and 5-year survival rates are estimated based on the depth of the tumor. Commonly, the higher the Breslow thickness, the worse the outcome[18].

- <1 mm: 5-year survival is 92-97%.
- 1 to 2 mm: 5-year survival is 80-92%.
- 2 to 4 mm: 5-year survival is 60-75%.
- >4 mm: 5-year survival is 50%.

This is a very accurate method for predicting the prognosis of melanoma, so it has been introduced into the standard TNM staging system for melanoma. The TNM staging is a globally recognized system that alphanumerically describes the stage of a cancer. It was developed by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC), and has been updated several times since then due to advances in melanoma research[17]. This system is used for many solid tumors and is based on the evaluation of three mandatory parameters:

- **T**: describes the size and extention of the primary tumor. It is based on the Breslow scale to determine tumor thickness and analyzes for the presence of ulceration.
- **N**: describes the spread of the tumor to nearby lymph nodes and if the metastasis present in the node is microscopic or macroscopic. Besides, it also identifies in-transit metastasis that are more than 3 cm away from the primary tumor but have not yet reached a lymph node, and satellite lesions, which are tumors larger than 0.5 mm that are located within the same histologic section as the primary tumor.
- **M**: describes the presence of distant metastasis.

Table 1. Description of the criteria used to classify the staging of melanoma[17].

Stage	Description
Tumor (T)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Melanoma *in situ*. Precancerous lesion. Melanoma cells are found between the epidermis and dermis of the skin, and have not invaded yet these layers.
T1	Tumor ≤1 mm thick
T1a	Tumor ≤1 mm thick, no ulceration and mitotic rate < 1/mm^2
T1b	Tumor ≤1 mm thick, either with ulceration or mitotic rate > 1/mm^2
T2	Tumor 1-2 mm thick
T2a	Tumor 1-2 mm thick, no ulceration
T2b	Tumor 1-2 mm thick, with ulceration
T3	Tumor 2-4 mm thick
T3a	Tumor 2-4 mm thick, no ulceration
T3b	Tumor 2-4 mm thick, with ulceration
T4	Tumor >4 mm thick
T4a	Tumor >4 mm thick, no ulceration
T4b	Tumor >4 mm thick, with ulceration
Node (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No melanoma found in regional lymph nodes
N1	Melanoma found in 1 lymph node
N1a	Melanoma found in 1 lymph node, microscopic metastasis
N1b	Melanoma found in 1 lymph node, macroscopic metastasis
N2	Melanoma found in 2-3 lymph nodes
N2a	Melanoma found in 2-3 lymph nodes, microscopic metastasis
N2b	Melanoma found in 2-3 lymph nodes, macroscopic metastasis
N2c	In-transit melanoma or satellite lesions are found, without metastasis to lymph nodes.
N3	Melanoma is found in ≥4 lymph nodes, or in ≥2 lymph nodes that appear to be joined together. In-transit melanoma or satellite lesions are found, with metastasis to lymph nodes.
Metastasis (M)	
Mx	Metastasis cannot be assessed
M0	No metastasis
M1a	Metastasis to skin, subcutaneous tissues or distant lymph nodes
M1b	Metastasis to lung
M1c	Metastasis to any other distant organs

After classification of melanoma according to the TNM system, an overall stage of the disease is assigned **(Table 2)**, where stage 0 is melanoma *in situ* and is recognized as precancerous. Stages I and II are considered localized lesions. Stage III corresponds to regional disease and Stage IV is considered as advanced disease[17,19].

Table 2. Staging of melanoma based on TNM system[17].

Stage	T	N	M
0	Tis	N0	M0
IA	T1a	N0	M0
IB	T1b T2a	N0	M0
IIA	T2b T3a	N0	M0
IIB	T3b T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T4a	N1a N2a	M0
IIIB	T1-T4b T1-T4a	N1a N2a N1b N2b N2c	M0
IIIC	T1-T4b Any T	N1b N2b N2c N3	M0
IV	Any T	Any N	M1

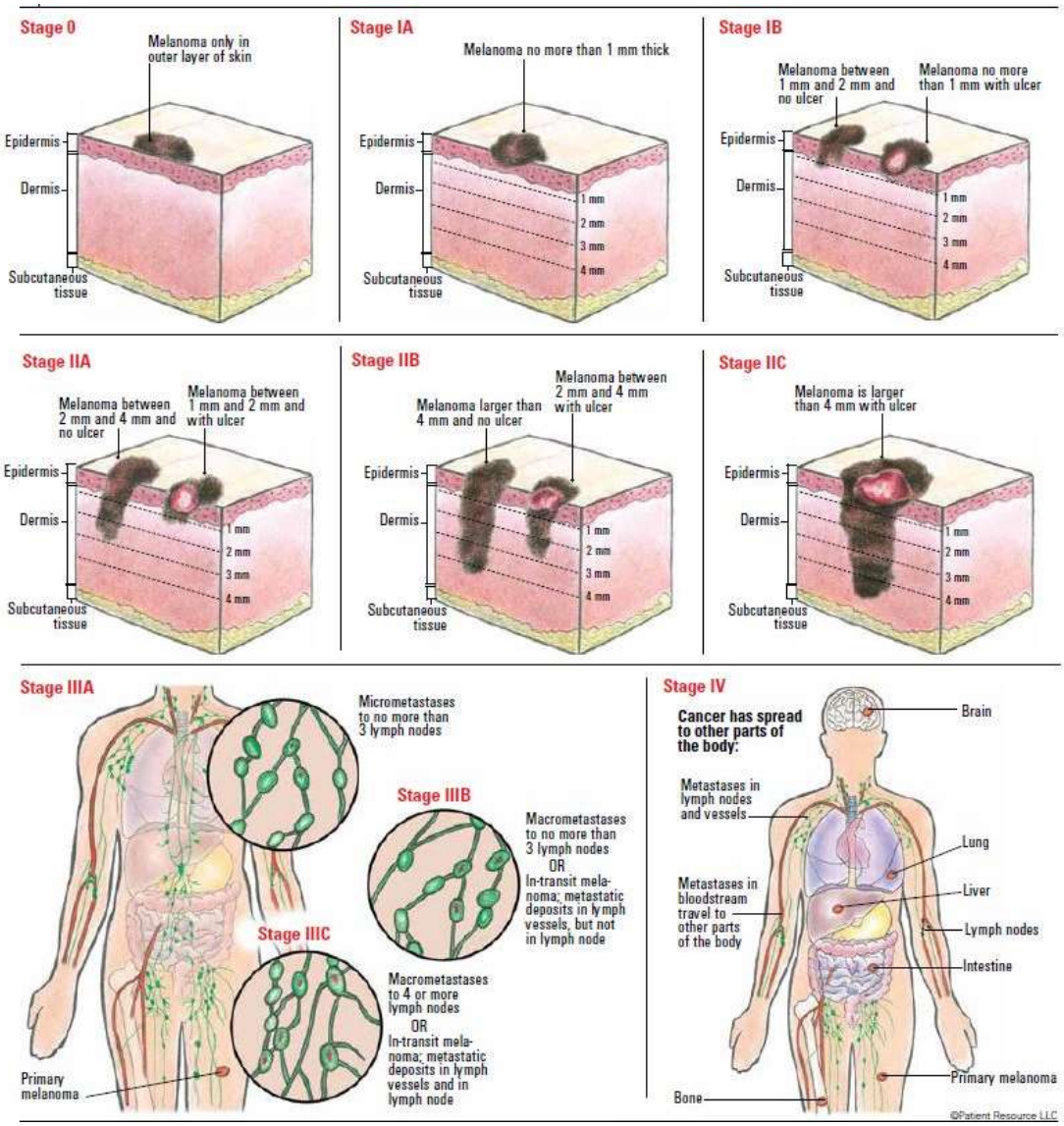


Figure 9. Clinical description of each stage of melanoma development[17].

1.3.4. Clinical classification of melanoma

Classically, four different clinical types of melanoma have been distinguished based on their anatomical localization and evolution.

- **Superficial spreading melanoma (SSM):**

It is the most common form of melanoma among Caucasians, representing 70-80% of all the melanoma cases in this ethnic group. It usually arises on sun-exposed skin, such as the trunk and back in males and the back and lower limbs in females. Besides, about 40% of SSM evolve from a pre-existing lesion such as a common or a dysplastic nevus. The clinical features of this lesion meet the ABCDE criteria, as it typically has irregular and asymmetrical edges, with color variation and is larger than 6 mm. This type of melanoma presents a prolonged radial growth phase, where the lesion remains thin as the melanocytes grow along the epidermis. However, if the tumor is not excised, it may eventually begin the vertical growth phase and invade the dermis and reach the hypodermis, compromising the patient's survival[19–21].

- **Nodular melanoma (NM):**

Despite being the second most common type of melanoma with an incidence of 15-25%, it is identified as the most aggressive form since it presents a quick growth that begins directly with the vertical growth phase. In addition, it develops *de novo*, not needing a pre-existing lesion, so its identification is more difficult and is usually diagnosed when it already has metastatic capacity. It regularly appears on the head, neck or trunk of middle-aged patients, and it is more common in men than in women. Macroscopically, it can be described as a fast-growing, dome-shaped, blue-black lesion, with high tendency to ulceration[20].

- **Lentigo maligna melanoma (LMM):**

It accounts for less than 10% of all melanoma cases. It originates from a pre-existing *in situ* melanoma called lentigo maligna that is found in the epidermis and grows peripherally; in fact, it can reach a diameter of 5-7 cm at this stage. Over a period of years, the tumor acquires the invasive phenotype and grows vertically; henceforth, it is called lentigo maligna melanoma. It arises in sun-exposed areas of sun-damaged skins, principally on the face and neck of Caucasian elders. It is a large lesion (>3 cm), multicolored (different shades of brown, black), and with elevated areas[20].

- **Acral lentiginous melanoma (ALM):**

Unlike the other variants, ALM is more frequent in black (60-70%) and Asian (35-45%) populations than in fair-skinned individuals (<5%). It occurs on non-hairy body surfaces such as palms, soles, and beneath nail beds, although most ALM develop on the soles of the feet of elderly individuals. Their appearance is similar to that of lentigo maligna melanoma, since they are also black, irregular and large.

In addition, there are other less common variants such as spitzoid melanoma, small cell melanoma, malignant blue nevus, desmoplastic melanoma, ocular melanoma (conjunctival or uveal) and mucosal melanoma, among others.

1.4. Melanoma treatment

The recovery and therapy election of melanoma patients is directly correlated to the stage at which it is diagnosed. While the recovery rate is almost 100% when diagnosed early, there is no utterly effective therapy for metastatic melanoma. The treatment of choice for localized melanomas in stage 0 to II is surgical excision with a wide margin to prevent local recurrence[22,23]. Moreover, if Breslow's thickness is high and there is metastatic suspicion, sentinel lymph node biopsy is carried out. This is defined as the first draining node of the primary tumor, so it would be the first to receive metastatic cells. This clinical approach allows the identification and subsequent excision of this lymph node, which is then histologically examined, and if metastatic cells are found, an immediate lymphadectomy is carried out.

Melanoma has long been considered a radioresistant tumor and the use of radiotherapy has been reserved for palliative treatment. However, recent findings suggest that radiotherapy can be applied as adjuvant therapy post-surgery or when the suggested excision margins cannot be applied in surgery, especially in elderly patients with lentigo maligna melanoma[24].

1.4.1. Metastatic melanoma

The treatment of metastatic patients remains the main obstacle to overcome in the management of melanoma. These patients have a very poor prognosis, and surgery and radiotherapy are not effective for advanced disease. Dacarbazine chemotherapy has been the first-line treatment from 1975 to 2011. Until recently, the only FDA-approved treatment for metastatic melanoma was dacarbazine, but temozolomide is also used as chemotherapy today. However, they exhibit low response rate and the effect lasts only 5 to 6 months[25–27].

A further treatment option for metastatic melanoma is immunotherapy, which uses the patient's own immune system to attack the tumor. The first immunomodulatory agent approved for melanoma was cytokine IL-2 in 1998. It is usually administered as an adjuvant therapy together with chemotherapy or radiotherapy. However, it is associated with significant toxicities, so its use is limited to patients in a good performance status[25]. Interferon-α (IFN-α) and peginterferon α-2b are also used although they have low antitumor response and are associated with severe side effects, so their use have been reserved to stage IV patients[25,28].

The revolution in the treatment of advanced melanoma began in 2011 when ipilimumab was approved for metastatic melanoma or as adjuvant therapy in patients with resected stage III melanoma. This humanized monoclonal antibody acts against cytotoxic T lymphocyte-associated protein 4 (CTLA-4), resulting in the secretion of IL-2 and activation and proliferation of cytotoxic T cells[25,28–30].

Other checkpoint inhibitors are the PD-1 pathway inhibitors. In 2015, the FDA approved nivolumab and pembrolizumab for their use in advanced melanoma or as adjunvant therapy after surgery. These monoclonal antibodies block the interaction between PD-1 receptor of T cells with PD-L1 antigen of melanoma cells, boosting the antitumor response of T cells. Interestingly, the combination of ipilimumab and nivolumab has been studied, but important adverse effects have been described[25,28–32].

Targeting therapy has become another treatment option for melanoma. In approximately 50% of cases, there is BRAF-V600E mutation. Therefore, the FDA and EMA (European Medicines Agency) have approved two different BRAF-V600E inhibitors to use in metastatic melanoma, namely vemurafenib, approved in 2011, and dabrafenib, in 2013. Although they have shown significant improvement in patients, they tend to generate resistance to the treatment and the

effect does not last long[29,33]. Additionally, the MEK inhibitors approved for monotherapy are trametinib and cobimetinib, which can be used with patients that have the frequent BRAF or NRAS mutations. Significantly, MEK and BRAF inhibitors can be combined generating a highly efficient synergistic outcome[25,29,33]. The combination therapies that are approved at this time are vemurafenib and cobimetinib; dabrafenib and trametinib[32,34].

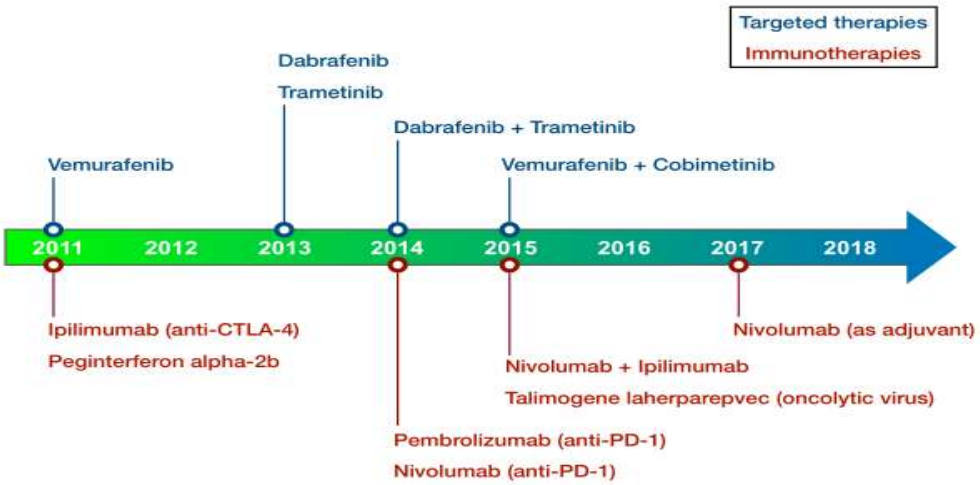


Figure 10. Timeline of melanoma treatment options since 2011[34].

1.4.2. New challenges

Lately, several clinical trials have been conducted to study the efficacy of new agents for the treatment of melanoma. Some of these new therapeutic approaches include the use of oncolytic viruses, being Talimogene laherparepvec (TVEC) the only approved option to date. Viruses replicate within cancer cells, generating GM-CSF and producing the lysis of neoplastic cells[29].

An interesting strategy under study are cancer vaccines, which stimulate the host's immune system to act against the tumor. The vaccines studied so far have been designed against antigens such as gp-100, Melan-A and tyrosinase. However, the response rates achieved have been low[35].

Another treatment option under research is adoptive cell therapy, which uses isolated tumor infiltrating lymphocytes (TILs) that have potent anti-tumor properties. TILs are expanded ex vivo and reinserted into the host to treat different metastases[35]. In another study, autologous lymphocytes were genetically engineered to detect and attack melanoma cells. Despite promising results, this treatment option needs further research to achieve more specific binding to tumor cells[36].

1.5. Melanoma biomarkers

In spite of the relevant advances recently achieved in the diagnosis and treatment of melanoma, there are no fully effective therapy or biomarkers for this disease.

A biomarker is an objectively measurable biological substance, structure, or process found in the body or in its waste products that indicates the risk to suffer a particular disease, the presence of a disease, the state of a disease and possible outcome, and determines the susceptibility to therapy. These biomarkers can be, for instance, genetic pathways or particular genes, metabolism routes, proteins or lipids that are modified after genetic, cellular, biochemical or molecular alterations generated by the carcinogenesis process[37].

An ideal cancer biomarker should be involved in the process by which the tumor develops, so it could also be a therapeutic target. Therefore, changes in its levels should be correlated with variations in the stage of the disease or with successful treatment. Moreover, it should not appear in healthy tissues or individuals, and should be specific for a concrete type of cancer. Ideally, this biomarker would be used to assess predisposition to a particular type of cancer, early diagnosis, prognosis, and drug response. Besides, it must be detected easily and non-invasively.

Particularly in melanoma, the diagnosis is based on clinical screening and identification, followed by histopathological confirmation after studying the subsequent histological biomarkers:

HMB45 antibody recognizes premelanosome protein (**Pmel**) that is present in melanoma and junctional nevus. However, it has shown little sensitivity for desmoplastic malignant melanoma.

Melan-A protein is present in the cytoplasm of both melanocytes and melanoma. It shows a high specificity to differentiate between melanoma and non-melanocytic tumors, although it is less sensitive when detecting metastatic melanoma and desmoplastic melanoma, compared with primary tumors.

Tyrosinase enzyme is implicated in the production of melanin and is very good at differentiating melanoma and non-melanocytic tumors. However, it also demonstrates a lower sensitivity to detect desmoplastic melanoma.

S100B protein is routinely used as an immunohistochemical biomarker for melanoma. Although it is not specific for melanoma, since it is also elevated in patients with kidney and liver damage, liver metastases from other types of tumors, and diverse infectious and inflammatory disorders. It presents high sensitivity for desmoplastic melanoma, so it is of great utility for pathologist.

Although these markers help in the confirmation of the histopathological diagnosis of melanoma, none of them is able to distinguish between malignant and non-malignant melanocytic lesions.

Additionally, there are other biomarkers that help determine which patients are likely to progress to more advanced stages or would benefit from a concrete treatment.

Although they can not be considered biomarkers, the **Breslow thickness** and the **mitotic rate** are the most precise factors to determine melanoma prognosis and survival of patients, and are part of the TNM staging system. In fact, tumors with high Breslow thickness and mitotic rate, together with absence of infiltrated lymphocytes are more likely to be positive for sentinel lymph node.

Moreover, the assessment of the presence of the **BRAF V600E** mutation helps to determine the suitable treatment for patients, since vemurafenib has been shown to significantly increase the survival of individuals who have this mutation.

In addition to histopathological markers, there are also serum biomarkers, which are detected less invasively. Among them, **lactate dehydrogenase** (LDH) enzyme is found. High levels of serum LDH are associated with metastatic melanoma, especially with liver metastases. In fact, it has been introduced in the TNM staging system as an adverse prognostic marker and a negative predictor of response to therapy. However, it is not exclusive for melanoma.

S100B protein can not only be considered a histological biomarker for diagnosis as explained above, but also a serum biomarker for advanced melanoma, since it is associated with metastatic melanoma, reduced survival, relapse and poor response to treatment[38].

Besides, there are other biomarkers that, although not exclusive of melanoma, are also used in the evaluation of prognosis, since they have increased levels in various inflammatory processes, such as tumor inflammation, angiogenesis and metastasis. These include VEGF, metalloproteinases and cyclooxygenase-2 enzyme, among others[39].

1.5.1. Biomarker discovery

The research process to discover new biomarkers can be divided into three different phases. The first one is based on basic studies and is called discovery phase. Here, the hypothesis and the analytical approach are defined, and the proposed assays are carried out. This is the phase where potential biomarkers are identified. In order to discover robust candidates, the analytical processes employed must be precise and reproducible, to ensure the reproducibility of the results across different laboratories.

In the second phase, the analytical validity and clinical validation of the biomarkers is studied. For this, the biomarkers are implemented in the platform that would be used in clinic. Then, new samples are studied and the biomarker must faithfully classify the samples in the appropriate validation groups.

A biomarker that has successfully fulfilled analytical and clinical validation is ready to determine its clinical utility. In this phase, clinical effectiveness is assessed together with the benefit-to-harm ratio. A biomarker that would be used in patient care must present high levels of clinical evidence. Besides, for clinical implementation, the biomarker must achieve regulatory approval, commercialization and incorporation into clinical practice guidelines[37,40].

1.6. Melanoma hallmarks

In order to identify new cancer biomarkers, the complex nature of tumors must be well understood. For this purpose, the hallmarks of cancer were defined, which comprise 10 different and complementary capacities acquired by different cancer cells to guarantee their survival, proliferation and dissemination, regardless of the cancer type[41]. This simplified conceptualization defines a framework for understanding the sophisticated biology of different types of cancers. They affect both tumor and stroma cells, and are established as follows:

- **Genomic instability:** In melanoma cells, UV radiation produces genomic instability, inducing the appearance of mutations, which allows the development of other cancer hallmarks and, therefore, tumor progression[42].

- **Tumor promoting inflammation:** Tumor tissues present chronic inflammation enabling the release of many signaling molecules from the immune cells. These factors promote proliferation and resistance to death, contribute to genomic instability and stimulate angiogenesis and metastasis[41]. In melanoma, concretely, UV radiation damages melanocytes and keratinocytes, which release HMGB1 cytokine that recruits neutrophils to the tumor microenvironment[42]. These immune cells release pro-inflammatory proteins that promote genome instability, angiogenesis and metastasis.
- **Sustaining proliferative signaling:** Cancer cells produce and release growth factors, and also increase the expression of the receptors for these ligands. In addition, they send signals to stromal cells in order to make them produce more growth supporting molecules. Besides, this can also be achieved independently from growth factors, by constitutively activating downstream proliferative signaling pathways. In melanoma, very often, this is achieved due to BRAF activating mutation, which keeps the RAS/RAF/MERK/ERK pathway continuously activated resulting in excessive proliferation[42].
- **Evading tumor growth suppressors:** Two major tumor suppressor proteins, p53 and pRb, arrest the cell cycle and induce senescence or apoptosis. In melanoma, deactivating mutations in tumor suppressor gene CDKN2A are common. It encodes p16 protein that can dephosphorylate pRb, and therefore, deactivate it and arrest cell cycle. If CDKN2A is mutated, p16 does not deactivate pRb, and the cell cycle is continuously on[42].
- **Resisting cell death**: Apoptosis is essential for the proper homeostasis of the tissues. If there is any defect in the apoptotic pathways the cells can acquire resistance to cell death. In cutaneous melanoma, defects in the apoptotic pathways have been reported. For instance, in metastatic melanoma Apaf-1 protein, which plays a key role in activating apoptosis, when cytochrome c is released from the mitochondria following DNA damage, is absence[42].
- **Enabling replicative immortality:** Cancer cells have the ability to replicate limitlessly. This is achieved by the upregulation of telomerase, which constantly adds telomeric DNA to the ends of the telomeres, so they are not shortened, and therefore senescence or apoptosis is not triggered[41].
- **Inducing angiogenesis**: angiogenesis is essential for tumor progression and dissemination. Melanoma cells release glycoproteins that aid in angiogenesis processes[42].
- **Activating invasion and metastasis:** Melanoma cells produce different proteins, such as metalloproteinases, that help in the degradation of the basal layer of the tissues and the extracellular matrix favoring the metastatic process[42].
- **Avoiding immune destruction**: Both innate and adaptive immune systems play a role in eradicating tumor cells. Some cancer cells, for instance melanoma cells, are able to escape immune detection and avoid immune destruction[42].
- **Deregulating cellular energetics**: In order to meet all the needs that cancer cells have, metabolism adapts to provide them with building blocks and energy to sustain the high proliferation and division. Melanoma cells switch their metabolism from oxidative phosphorylation to glycolysis, and the glucose uptake is significantly upregulated[42]. Besides, other anabolic and catabolic pathways are also altered in the cancer cells. Among these, lipid metabolism has gained striking importance over the last years, as it will be explained in the next section.

2. Lipids, cell metabolism and cancer

2.1. Lipids

Lipids are ubiquitous molecules that participate in a wide variety of vital physiological processes in the cells. In short, they are involved in cellular signaling, energy storage, structural support in biological membranes, and synthesis of physiologically important molecules, such as bile acids, some hormones and vitamins. They therefore control cellular processes such as proliferation, migration, survival and death, all of which are closely related to the carcinogenic process.

2.1.1. Lipid classification

In spite of existing beyond thousands of different lipid molecules, they all share some common features such as poor or no solubility in water, but good solubility in polar solvents. Generally, they are described as either hydrophobic or amphipathic molecules. Based on their molecular structure, they can be categorized into eight classes: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides.

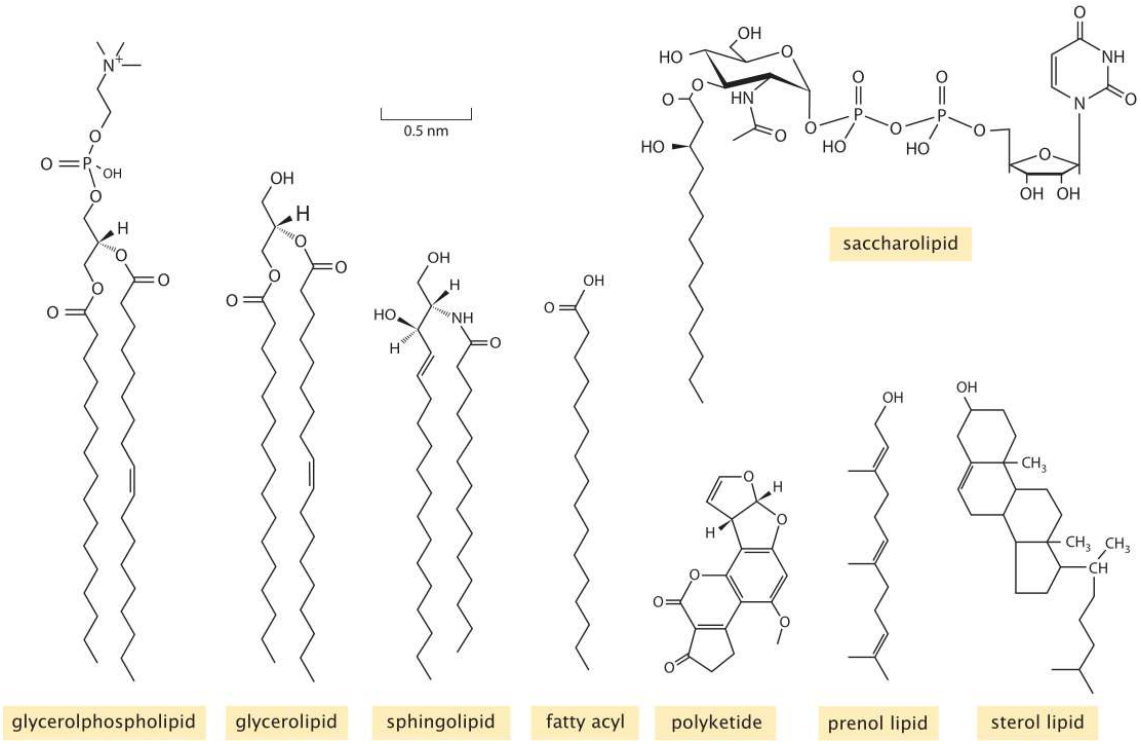


Figure 11. General structure of the different lipid classes[46].

Fatty acids (FA) are considered the simplest lipid molecules and one of the most fundamental classes of biological lipids, as they serve as building blocks for more complex lipids. Their structure can be divided in two parts; a non-polar hydrocarbon chain of different lengths, mostly with an even number of carbon atoms, and a polar carboxylic acid group at the end. In addition, there can be double and triple bonds within the hydrocarbon chain, forming monounsaturated FAs (one double or triple bond; MUFA) or polyunsaturated FAs (more than one insaturation; PUFA). If there is no insaturations, these FAs are named saturated FAs. The presence of insaturations in the acyl-chains creates a bend in the structure of the molecule. There have been identified over 1000 FA structural variants, by the combination of different chain lengths,

number and position of insaturations, and the presence of additional substituents along the hydrocarbon chain, such as sugar molecules. Nonetheless, the more common FAs in nature are around 20, with palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2) making up around the 80% of the most ordinary FAs[43].

Table 3. Short description of the three most common fatty acid molecules.

Fatty acid	N. of carbons	N. of insaturations
Palmitic acid	16	0
Oleic acid	18	1
Linoleic acid	18	2

FAs can be synthetized *de novo* inside the cell or taken up from the extracellular space[44]. The absorption of FA by the cells can be divided in two different mechanisms: transporter mediated up-take, by CD36 fatty acid translocase among others, and passive permeation[45]. There are essential fatty acids that can not be synthetized and are fundamental for some biological functions, so they need to be taken up from the diet. These are polyunsatured FAs with the double bonds close to the methyl end, and are divided in two series: omega-3 series based on linolenic acid with the double bond between the position C3 and C4, and omega-6 series based on linoleic acid with the double bond between the C6 and C7 position. After the acquisition of these lipids, there are different metabolic modifications catalyzed by elongases and desaturases and these lipids are transformed to other FAs, such as eicosapentaenoic acid (20:5) (EPA; omega-3) and arachidonic acid (20:4) (AA; omega-6)[46].

On the other hand, FAs can be newly synthetized by condensation of two-carbon units provided by malonyl-CoA and acetyl-CoA molecule, by the action of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) enzymes, yielding palmitic acid (16:0). For the synthesis of other FAs, starting from the newly formed palmitic acid or externally absorbed FAs, the acyl chain of these lipids can be further elongated and desaturated. The elongation of the carbon chain implies the action of different elongases (ELOVLs) that cyclically add two-carbon units. Moreover, the FAs may have a straight saturated chain, or can contain one or more double bonds or insaturations. Mammalian cells contain specific desaturases that introduce double bonds in a defined position of the long-chain FAs. There are two different families of desaturases: stearoyl-CoA desaturases (SCD) introduce a double bond in C9 of the carbon chain, whereas FA desaturases (FADS) introduce the double bond in C5 and C6[47].

Once the FA is synthetized, it needs to be covalently bound to a CoA by the action of fatty acyl-CoA synthetases (ACS), in order to enter the bioactive lipid pool of the cells. FAs have two main functions. On the one hand, they can be incorporated to other molecules forming more complex lipids. The fabricated lipids can be used for several purposes within the cells: precursor of lipid signaling molecules, store energy by generating triglycerides, fabricate phospholipids for structural purposes in the cell membranes and modify proteins through palmitoylation, among others[48,49]. The combination of different FAs in the structure of more complex lipids generates the structural variants of these complex lipids, changing their biological function. Some biologically important FAs act also as signaling molecules. Among them, the oxidation of arachidonic acid (20:4) or other polyunsaturated FAs (PUFAs), gives rise to eicosanoids, including

prostaglandins, leukotrienes and thromboxanes, with dramatically important biological functions[44].

On the other hand, they can also be used as fuel after their oxidation in the mitochondria.

Glycerolipids (GL) are formed by the esterification of one, two or three FAs to a glycerol backbone, forming mono- (MG), di- (DG) or tri-glycerides (TG), respectively. They function mainly as energy stores since the excess of energy in the body is kept in TGs containing 3 FAs for further oxidation when required. Moreover, DGs are signaling molecules. They are second messengers as they participate in the transmission of signals across the cellular membranes and participate in the biosynthesis of TGs and glycerophospholipids[50].

Glycerophospholipids (GPLs) are the main components of cell membranes, as well as important actors in cell metabolism and signaling. These lipids contain a glycerol backbone, with two FAs bond in sn-1 and sn-2. Frequently, GPLs contain a saturated FA at sn-1 and an unsaturated FA at sn-2. In the third carbon of the glycerol there is a phosphate followed by different polar heads, and based on the nature of which GPLs are divided into different subclasses. Therefore, they are amphipathic molecules, since they have a polar head group and two hydrophobic hydrocarbon tails. Depending on the polar head, and the length of the carbon chains and the number of insaturations of the fatty acids, there are variations on the biological function of the lipid and the physicochemical properties of the membranes where these lipids are located.

- Phosphatidic acid (PA) or phosphatidate is the intermediary for the synthesis of other glycerophospholipids and triacylglycerols, so it is present in low amounts in the tissues. It is formed by a glycerol molecule with two FAs esterified in sn-1 and sn-2, and in the third carbon, it has phosphoric acid. Thus, the polar group of PA is a hydrogen. At least four distinct pathways can generate PA. The main one takes place in the endoplasmic reticulum or mitochondria, GPAT (glycerol-3-phosphate acyltransferase) performs two sequential acylations in the sn-3-glycerol-3-phosphate that is generated in the catabolism of glucose. Another biosynthetic route of PA is the hydrolysis of other GPLs, yielding PA and the free polar head. For instance, Phospholipase D1 and D2 enzymes control the conversion of phosphatidylcholine lipids into PA and free choline[51,52].
  In addition to being a precursor of other lipids, overwhelming evidences reveal its role as a signaling molecule. Indeed, its presence affects cell growth, proliferation, motility and survival[51,52].

- Phosphatidylcholine (PC) has choline as the head group. Frequently, PCs contain palmitic acid (16:0) or stearic acid (18:0) molecules in sn-1, and unsaturated oleic acid (18:1), linoleic acid (18:2) or linolenoic acid (18:3) in sn-2[52,53].

  Choline is an essential nutrient for humans, with very high requirementsfor pregnant and lactating women, as it cannot be synthetized and is taken from the diet. The human body requires choline to synthetize phosphatidylcholine, choline ether lipids, and sphingomyelins; all these lipid subclasses are fundamental structural components of cell membranes. Indeed, the uptake of choline is mediated by choline transporters and it is mainly converted to PCs, which are very abundant in the lipid bilayers, particularly in the outer side, representing around 50% of all the GPLs in the membranes[54]. The presence of unsaturated FAs in the structure of PCs confers fluidity to the cell membranes. Interestingly, in order to fabricate the strikingly important acetylcholine neurotransmitter choline is also needed.

Although there are additional routes, the predominant PC synthesis pathway is the Kennedy pathway **(Fig. 12)**. Here, a choline that is transported inside the cell is phosphorylated by choline kinase (CK) in the cytoplasm, and activated by the addition of cytidine triphosphate (CTP) via CTP:phosphocholine cytidylyltransferase (CCT). The formed CDP-choline is transported to the endoplasmic reticulum, where, eventually, CDP-choline reacts with a lipid anchor, a diglyceride molecule, yielding a PC. Another synthesis route to produce PCs takes place in the liver mainly, where PE methyltransferase (PEMT) catalyzes three sequential methylations of the ethanolamine moiety of phosphatidylethanolamine (PE), yielding a PC[55] **(Fig. 12)**. After their synthesis there is a remodeling process called Lands' cycle **(Fig. 14)**. This is a strikingly important metabolic process common to all GPLs, where after their synthesis their FA chains are released by fosfolipase A_2 (PLA_2) enzyme and a different FA is incorporated by reacylation.,

On the other hand, there are several catabolic routes for PCs. Phospholipase enzymes catalyze the hydrolysis of the different GPLs, as it will be further explained in section 3 (page 46). Phospholipase D family enzymes hydrolyze PCs releasing PA and choline. This choline is frequently used for the production of new PCs. In addition, via phospholipase C, DG and phosphocholine are generated, and via phospholipase A family of enzymes (PLA_1 and PLA_2) a free fatty acid and lyso-phosphatidylcholine are produced. All the byproducts of these catalytic routes have extremely important roles as signaling molecules.

Interestingly, PCs are precursors for the synthesis of other lipids, including sphingomyelins, platelet-activating factor and PEs, all of them playing important structural and signaling roles[53,55].

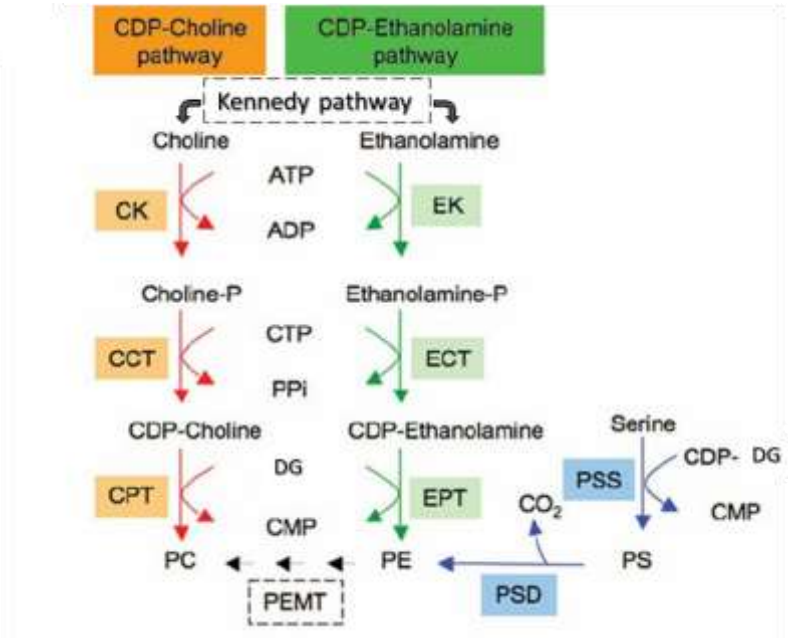


Figure 12. *De novo* synthesis pathways of PC, PE and PS. Adapted from [61]. Abbreviations: CK –choline kinase, CCT –CTP:phosphocholine cytidylyltransferase; CPT –choline phosphotransferase; EK –ethanolamine kinase; ECT –CTP:phosphoethanolamine cytidyltransferase; EPT –ethanolamine phosphotransferase; PEMT –PE methyltransferase; PSS –phosphatidylserine synthase; PSD –phosphatidylserine decarboxylase; ATP –adenosine triphosphate; ADP –adenosine diphosphate; CTP – cytidine triphosphate; PPi –pyrophosphate; DG –diglyceride; CMP –cytidine monophosphate; CDP-DG – cytidine diphosphate diglyceride.

- Phosphatidylethanolamine (PE) contains ethanolamine as the polar group, and is the second most common GPL in mammals. PEs are present in every cell of the human body, representing about 25% of all the GPLs, while in nervous tissue they can make up to 45% of all GPLs. Moreover, its abundancy varies from one organelle to another, accumulating greater amounts in mitochondria. Like PCs, they commonly contain palmitic acid (16:0) or stearic acid (18:0) in sn-1, but a longer unsaturated FA (20-22 C) in sn-2. They are also major components of the cell membranes, and can be found mainly in the cytoplasmic side of the lipid bilayers. However, they are present in both the inner and outer leaflet of mitochondria membrane, where they exert fundamental functions. In particular, they facilitate the protein movement across the membranes and membrane fusion. In addition, they are precursors of 35% of PCs synthetized in the liver. In the brain, they are precursor for the synthesis of anandamide, a cannabinoid ligand. Moreover, they are important for mitosis as they control the fusion of mitotic Golgi membranes. They are also significant in autophagy. Thus, they are important not only for their structural function but also for some fundamental cellular processes. Furthermore, they act as growth factor in cell cultures and have been proven to overcome the apoptosis produced by low serum culture conditions[26,56,57].
  PEs can be synthetized through different pathways. Like for PCs, Kennedy pathway is the principal route **(Fig. 12)**, albeit ethanolamine is the substrate and its phosphorylation is performed by ethanolamine kinase (EK). In the second step, CTP is condensed with the phosphoethanolamine by the action of ECT enzyme (CTP:phosphoethanolamine cytidylyltransferase), yielding CDP-ethanolamine. In the final step, EPT (ethanolamine phosphotransferase) catalyzes the reaction between CDP-ethanolamine and a lipid anchor, normally DG, giving rise to PE. The *de novo* synthetized PEs are mainly mono- or di-unsaturated acyl chains at the sn-2 position, such as 18:2[26,55].
  The second source of PE is the mitochondria, where phosphatidylserine decarboxylase (PSD) decarboxylates a PS molecule yielding a PE **(Fig. 12)**. Another minor synthesis route for PE takes place in the ER, and is a calcium-dependent base-exchange reaction where the serine of a PS is substituted by an ethanolamine[56,57]. As well as PCs, after the synthesis of PEs, they are remodeled in Lands' cycle **(Fig. 14)**.
  However, mammals cannot synthetize ethanolamine, so it is acquired from the diet as free ethanolamine, or from existing PEs, which are hydrolyzed by phosphodiesterases to produce free ethanolamine and glycerol. Another source of ethanolamine is the degradation of sphingosine phosphate by sphingosine phosphate lyase and the lysis of the endocannabinoid anandamide by the fatty acid amine hydrolase[56].

- Phosphatidylserine (PS) has a serine head group and accounts for the 3-15% of all the GPLs. However, the brain and retina are enriched with PS. There is also different concentrations of PSs within organelles, being abundant in plasma membranes, specially the inner leaflet, and scarce in mitochondria inner membrane[58].
  In mammals, the synthesis of these lipids is carried out by the calcium-dependent substitution of a polar group (choline or ethanolamine) for a serine, in preexisting PCs or PEs. Conversely, PSs can be decarboxylated in the mitochondria producing PEs.
  The main functions of PSs are being the precursors of PE in mitochondria, targeting some proteins to the phagosomes, and modifying the catalytic activity of some enzymes, such as Annexin V and protein kinase C. To gain insight into the fundamental role of PSs in mammalian cells, a study unraveled that mutant mice with impaired PSs synthesis did

not outlast during development. Indeed, PS play a role in blood clotting and apoptosis[57–59].

- Phosphatidylinositol (PI) carries an inositol molecule as polar head group, which is a cyclic hexalcohol. Essentially, they contain stearic acid (18:0) at sn-1 and arachidonic acid (20:4) at sn-2. They are structural components of the outer leaflet of cell membranes, making up around 10% of all GPLs, and aid in anchoring proteins to the outer surface of the cells, playing a strikingly important role in cell signaling[60].
  The *de novo* biosynthesis of PIs is carried out in the PI cycle **(Fig. 13)**, similar to the Kennedy pathway of PCs and PEs. First, CDP-DG is conjugated with inositol, catalyzed by phosphatidylinositol synthase, in the endoplasmic reticulum. Then, the formed PI can be remodeled by Lands' cycle, where there is a PI specific acetyltransferase, LPIAT (lyso-PI acyltransferase). PIs are the primary source of arachidonic acid (AA) that is needed for eicosanoid biosynthesis. [52,60,61].
  As part of the PI cycle, the inositol ring can be reversibly phosphorylated in the positions 3, 4 and/or 5 yielding 7 different phosphoinositides (PPIn). These can be phosphatidylinositol phosphate (PIP), phosphatidylinositol biphosphate (PIP_2) or phosphatidylinositol triphosphate (PIP_3), that are very important signaling molecules. Although they represent only around 1% of all the GPLs in cell membranes, they can be second messengers or modulate the anchorage and activity of different membrane proteins, actively participating in several basic cellular processes, such as signal transduction, membrane dynamics, cytoskeleton reorganization, membrane and vesicular trafficking[52,62].
  Moreover, they have been largely investigated, since the phosphorylation of these molecules is driven by PI3K (phosphatidylinositol 3-kinase), which is mutated in several cancers leading to an aberrant activation of these enzymes. Moreover, PTEN lipid phosphatase that is responsible for the dephosphorylating of PI3Ks, thus inactivation of the enzymes, is also frequently mutated in several cancers. These results in increased PIP_3 levels, trait that has been strongly related to elevated metastatic capacity. Therefore, both PI3Ks and PTEN are among the most promising drug targets in cancer[52,62,63].

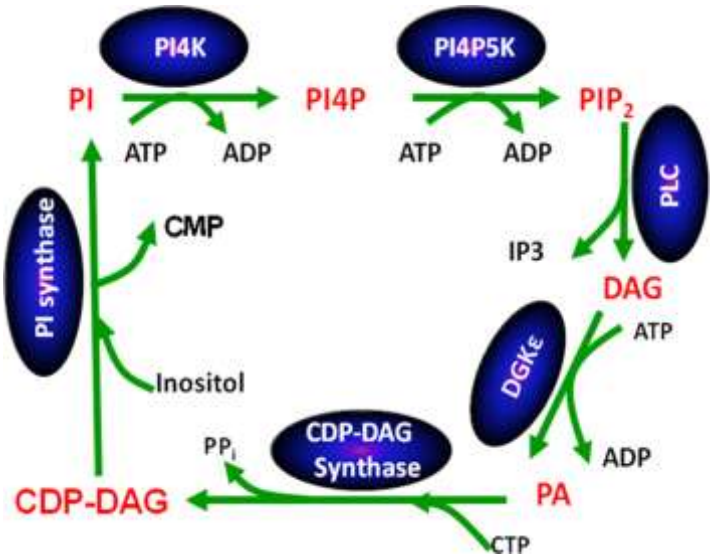


Figure 13. PI cycle. In blue ovals are the enzymes that catalyze each reaction of the route. In red are the lipid intermediaries[61].

- Phosphatidylglycerol (PG) is formed by a glycerol-3-phosphate backbone that has two FAs esterified in sn-1 and sn-2, and another glycerol molecule as the head group. It is commonly found in the inner mitochondria membrane. Although it appears at a low abundance in the membranes (1% of all PLs), its main function is being the precursor of mitochondrial cardiolipins.
  The *de novo* biosynthesis of this subclass is carried out by the condensation of CDP-DAG and glycerol-3-phosphate catalyzed by phosphatidylglycerolphosphate synthase, yielding phosphatidylglycerol-phosphate. Then, the intermediary is dephosphorylated with phosphatidylglycerolphosphate phosphatase producing PG phospholipid. Like in the rest of GPLs, the FAs of PGs can be remodeled in Lands' cycle. Concretely, a PG specific acyl-transferase has been found, LPGAT1, which shows a preference for 16:0, 18:0 and 18:1 FAs. As opposed to other GPLs, they often have more unsaturated FAs in sn-1 position[64,65].
  Due to the low abundance of these GPLs in mammal cells, little is known about their physiological and pathological functions.

- Cardiolipins have a unique dimeric structure as they are formed by a central glycerol molecule with two phosphatidic acids attached. Therefore, they content four FAs, which are commonly mono- or di-unsaturated chains with 16-18 carbons. Moreover, the distribution of the carbons and the double bonds is homogeneous within the four-acyl chains, since almost half of the cardiolipin structures studied are symmetrical. They are mainly found in the inner mitochondria membrane, making up to 20% of all the GPLs of this structure[66]. Here, they are important for membrane fusion and maintaining the enzymatic function of some enzymes of the electron transport chain, thereby influencing the energy production of the cells and acting as a proton trap during OXPHOS[67]. Besides, they trigger the apoptosis machinery when they are translocated to the outer side of the mitochondrial membrane[68,69].

- Ether phospholipids present an alkyl or alkenyl/vinyl bond between the glycerol and a fatty alcohol in sn-1, instead of an ester bond with a fatty acid. There can be ether lipids of all the glycerophospholipid subclasses, although choline and ethanolamine are the most abundant. In particular, plasmalogens are the most abundant ether lipids, and carry a double bond next to the ether bond, therefore forming a vinyl ether bond. Commonly, the fatty alcohols in sn-1 position are 16:0, 18:0 and 18:1, whereas polyunsaturated FAs concentrate in sn-2 position, generally docosahexaenoic (22:6) or arachidonic acid (20:4). Alkyl ether bonds are commonly found in ether phospholipids containing choline, and vinyl ether bonds are more prominent in ethanolamine plasmalogens[70,71].

  Their presence is very variable and depends on the tissue or organ, representing around 20% of all the glycerophospholipids, increasing their abundancy in heart, nervous tissue and inflammatory cells. According to the subcellular localization, they are enriched in plasma, nucleus, endoplasmic reticulum and mitochondria membranes. The main functions of these lipids are structural support in the nucleus and mitochondria, and cell vesicle formation, membrane fusion and ion transport. The presence of plasmalogens in biological membranes generates a tighter packing of the lipids forming the bilayer, thereby increasing rigidity and decreasing fluidity. Moreover, they have been found enriched in the lipid rafts of the bilayers, and the accumulation of these lipids together with sphingolipids and cholesterol was associated with increased stability of the membrane rafts. Indeed, several studies unraveled that the blockage of plasmalogen presence in the membranes is translated into oxidative stress and cell apoptosis, highlighting the importance of these lipids in the membrane homeostasis. In addition, they also have important signaling roles and antioxidant scavenging properties. Indeed, the vinyl-ether bond is able to capture reactive oxygen species and avoid the oxidation of the so sensitive PUFAs. Besides, they generate less persistent oxidative byproducts compared to other phospholipids[70,72–74].

  Ether lipids are synthetized by unusual and complex biosynthetic pathways that start in peroxisomes and finish in the endoplasmic reticulum. The peroxisomes are significant metabolic organelles as they participate in strikingly important metabolic processes including oxidation of branched and vey-long-chain FAs, ether phospholipids synthesis, bile acid synthesis, amino acid catabolism, polyamine oxidation, and the oxidative arm of pentose phosphate pathway (PPP)[72,74,75].

  For the synthesis of ether phospholipids, first, inside the peroxisome, dihydroxyacetone phosphate (DHAP) is esterified at sn-1 with a long-chain acyl-CoA, what is carried out by dihydroxyacetonephosphate acyltransferase (DHAPAT). Then, the ether bond is introduced via alkyl-DHAP synthase (ADAPS), by the substitution of the acyl of acyl-DHAP with a long-chain fatty alcohol. The remaining catalytic reactions take place in the endoplasmic reticulum, so 1-alkyl-DHAP is transported to that organelle. The first reaction in the ER is the reduction of the ketone group to form a glycerol, and an acyl group is introduced at sn-2, yielding a 1-alkyl-2-acyl-glycero-3-phosphate. Afterwards, the phosphate group is removed by a phosphohydrolase, and upon the activity of different enzymes the intermediary is transformed to either ethanolamine or choline ether lipids, in a process similar to the Kennedy pathway. As well as PCs, ether-PCs can also be synthetized by the methylation of ether-PEs through PEMT enzyme in the ER and mitochondria. At this point, the produced ether lipids are transported from the endoplasmic reticulum to the other organelles and plasma membrane, distributing

ethanolamine plasmalogens within the inner leaflets of the membranes and choline ether lipids within the outer side[65,66,71].

Ether lipids are also remodeled by phospholipases, as there is an ether lipid-specific PLA_2 isoform. In this way, ether lipids are considered important signaling lipids, since they usually contain AA, docosapentaenoic acid or docosahexaenoic acid (DHA) in sn-2, which are released upon PLA_2 activity[70–72].

The different glycerophospholipid subclasses are synthetized through specific *de novo* pathways that are summarized in **Figure 14.** This *de novo* biosynthesis takes place in the ER, peroxisomes and mitochondria, although the final remodeling steps can occur in either the ER or mitochondria. Starting from a glycerol molecule that is phosphorylated to glycerol-3-phosphate, a lysophosphatidic acid (LPA) is formed following the binding of a FA by glycerol-3-phosphate acylCoA:glycerol-3-phosphate acyltransferases (GPATs), which prefer saturated or mono-unsaturated FAs. Then, a second FA is esterified in the LPA yielding PA via acyl-CoA lyso-PA acyltransferases (LPAATs). The formed PA is further metabolized to form two different glycerol derivatives. One, DAG (or DG, they are named interchangeably in this work), is formed after the dephosphorylation of PA by phosphatidic acid phosphatase (PAP). This DAG is intermediary for the synthesis of TG by the addition of an acyl chain by diacylglycerol acyltranferase (DGAT) or by the transfer of an acyl chain from PC to DAG by phospholipid:diacylglycerol acyltransferase (PDAT). In addition, PC, PE and PS are also produced from DAG. PS is also formed from PC and PE. The other glycerol derivative is cytidine diphosphate-DAG (CDP-DAG) that is metabolized to form PI, PG, CL or PS.

The biophysical properties of the cell membranes depends on the characteristics of the phospholipids within these structures. This lipid variety is observed among different tissues and even between organelles within the same cell, being extremely important since it is reflected in the specific function of each compartment. Concretely, the lipid diversity is given by the distinct head groups, chain length and insaturations of the acyl chains that determine the fluidity, permeability, stability, curvature and subdomain architecture of the bilayers. It is reflected in the vesicular trafficking, signal transduction and molecular transport of the cells. Therefore, the phospholipid content of the membranes directly affects all these cellular processes. The remodeling process of the *de novo* synthetized GPLs is performed in Lands' cycle. As the enzymes of *de novo* synthesis pathways have little substrate specificity for concrete FAs, the generated GPLs may not contain the acyl chains suitable for a correct function of the membranes. Hence, the glycerophospholipids modify their FA chains by a series of deacylation and reacylation reactions. The saturated FA esterified in sn-1 position are believed to be generally derived from *de novo* synthesis, whereas the unsaturated FA that is generally observed in the sn-2 position is introduced in this remodeling process. For this, phospholipase A2 (PLA_2) specifically, cleaves the acyl chain at sn-2 of the GPL. Thus, important signaling molecules are generated, as lyso-phospholipids and free fatty acids are released, including the so important arachidonic acid (20:4). The latter can be converted to eicosanoids, which participate in a plethora of physiological and pathological processes, including inflammation, immune response, sleep regulation and pain perception. Then, the lyso-GPL can be reacylated by incorporating a different fatty acid to the previously cleaved position and forming a new phospholipid via specific acyl-CoA:lysophospholipid acyltransferases (LPLAT), as are different enzymes specific for each PL subclass. Therefore, this process can replace oxidized fatty acids and helps to generate

a diverse and asymmetrical content of acyl chains within the cell membranes, which is required for the correct function of the bilayers[76–79].

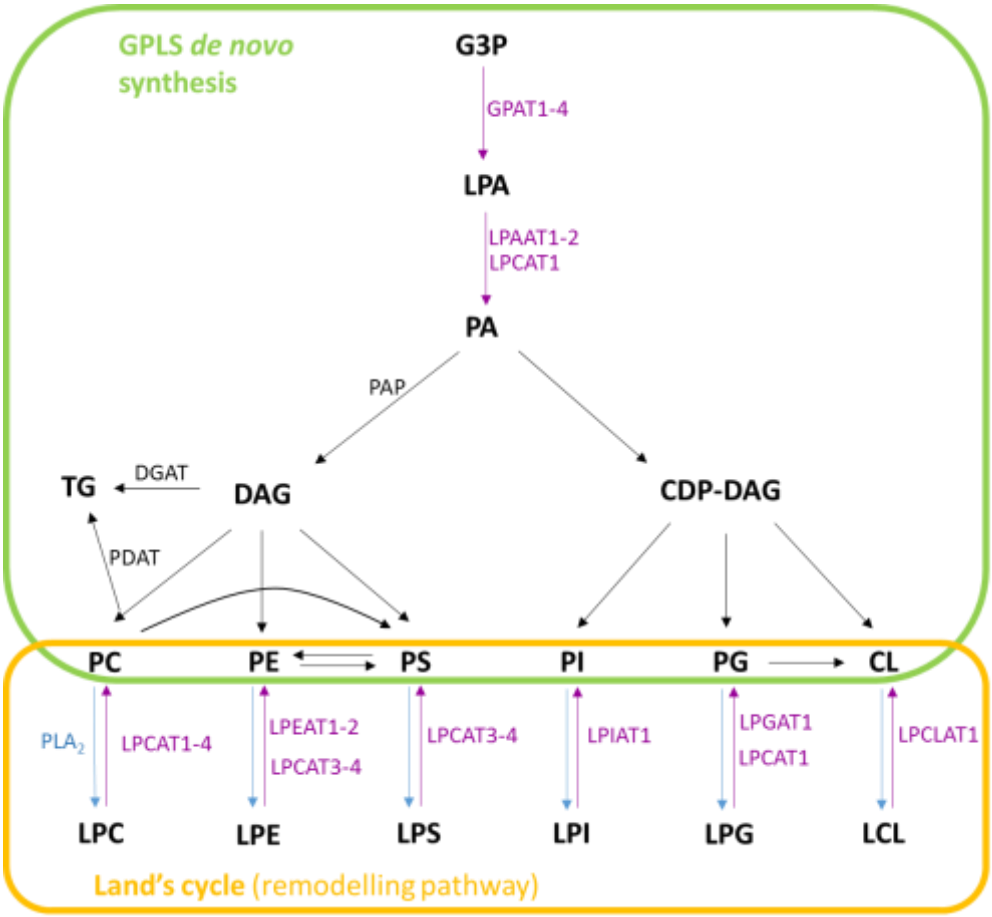


Figure 14. Glycerophospholipid biosynthesis. The combination of the different *de novo* synthesis pathways and Lands' cycle gives rise to phospholipid diversity. The blue arrows indicate PLA_2 and purple arrows acyltransferases. G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LPA, lyso-PA; LPAAT, lyso-PA acyltransferase; LPCAT, lyso-PC acyltransferase; CDG-DAG, cytidine diphosphate-DAG; LPE, lyso-PE; LPC, lyso-PC; LPS, lyso-PS; LPI, lyso-PI; LPG, lyso-PG; LCL, lyso-CL; LPEAT, lyso-PE acyltransferase; LPIAT, lyso-PI acyltransferase; LPGAT, lyso-PG acyltransferase; LPCLAT, lyso-CL acyltransferase.

Sphingolipids (SPLs) are a family of complex lipids that share a common structural feature, a sphingoid base backbone, which is composed of a set of aliphatic amino alcohols. The most common one is sphingosine, an 18-carbon length aliphatic chain with a double bond in position 4, hydroxyl groups in positions 1 and 3, and an amine group in position 2. The sphingoid bases are produced *de novo* by the condensation of the amino acid serine and a long-chain fatty acyl-CoA of different lengths and number of insaturation. Sphingosine is the most common sphingoid base and is synthetized by the condensation of serine with palmitic acid. In addition, the FAs that are bound to the sphingoid bases in this lipid class differs slightly from the ones of the GPLs, since they can be long chains of up to 26 carbons, and can be odd- or even-numbered. However, in the sphingolipids of the epidermis there can be FAs of 28 to 36 carbons. Besides, polyunsaturated FAs are rarely found within these lipids[80].

There are different bioactive lipids within sphingolipid family, such as ceramides, sphingosine 1-phosphate (S1P), sphingosine and sphingoglycolipids. They participate in different cellular processes closely related to carcinogenesis, as they regulate inflammation, senescence, apoptosis, angiogenesis, cell proliferation, survival, migration and differentiation[63].

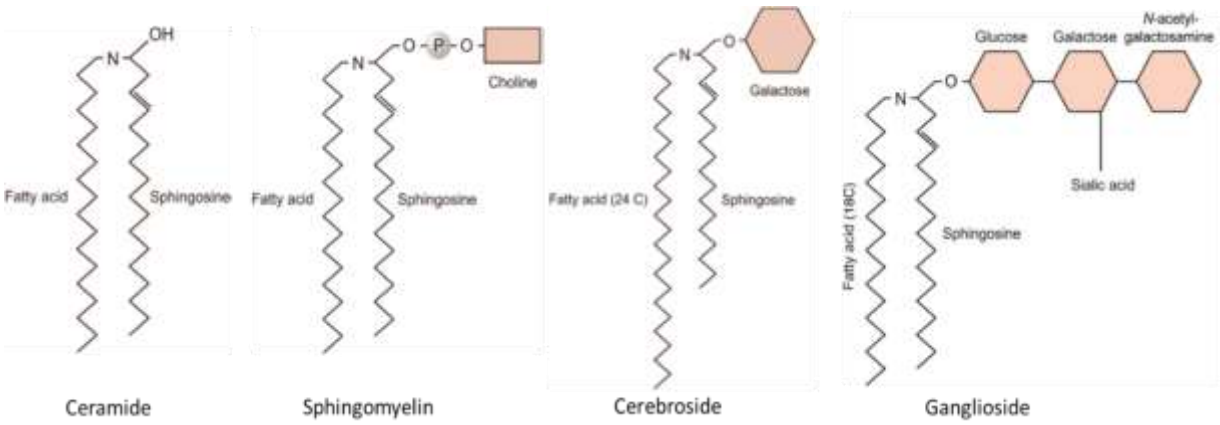


Figure 15. Schematic representation of the general structure of sphingolipids[54].

- Ceramides (Cer) are formed by the addition of a fatty acid to a sphingoid base through an amide bond. The acyl-chains of these molecules can be very distinct depending on their biological origins, although they are usually saturated or mono-unsaturated acyl chains. Despite their very low abundance in tissues others than the skin, they have important biological functions on their own, since they are important lipid second messengers. Additionally, ceramides can be precursors of more complex sphingolipids, which perform a wide range of functions in cells[81]. Furthermore, ceramides perform structural functions in the membranes, as they affect the permeability of the bilayers by interacting with the ion channels of the membranes. Although ceramides are minor components of the membranes, they are accumulated in rafts participating in several signaling events, generally activating catabolic enzymes and slowing down anabolic processes. Thus, they are significant players in the regulation of cell transformation, differentiation, proliferation, migration, cell death, autophagy, senescence and apoptosis. Indeed, the mechanism of action of many chemotherapeutic agents and radiation treatments includes the ceramide-mediated induction of cell death[81–83].
  Interestingly, there is a great accumulation of ceramides in the stratum corneum of the skin, making up to 50% of all the lipids found in this structure. Here, ceramides exist as both free molecules and esterified to structural proteins. Moreover, they present distinct features, as they contain larger carbon chains, which help them to participate in the barrier properties of the skin, since they confer lower permeability to the skin[82].
  The biosynthesis of ceramides is complex, and can be performed by distinct pathways **(Fig. 16)**. First, they can be *de novo* synthetized in the endoplasmic reticulum. It begins with the condensation of a serine and palmitoyl-CoA and subsequent CoA release by serine palmitoyltransferase (SPT), yielding 3-keto-dihydrosphingosine. Next, this is reduced by 3-ketosphinganine reductase to form sphinganine (dihydrosphingosine). The latter is condensed with a very-long chain FA, turning it to dihydroceramide by the action of dihydroceramide synthase (CerS). There are six different isoforms of CerS,

depending on the carbon-chain length of the ceramide they synthetize. For instance, CerS3 participates in the synthesis of very-long-chain (28-32 C) ceramides with polyunsaturated FAs, that are highly expressed in the skin[84]. Finally, a double bond is introduced in the position 4 of the sphingoid base via a dihydroceramide desaturase, to form the final ceramide. While most of the ceramide needed as intermediary is synthetized in the cytoplasmic leaflet of the endoplasmic reticulum, the production of more complex SPLs is carried out in the Golgi apparatus. Therefore, ceramides are transported to the Golgi apparatus by a key cytoplasmic protein, the ceramide transporter (CERT)[82,85,86].

Another route for ceramide biosynthesis is the catabolism of complex SPLs inside the lysosomes, the route called salvage pathway. This route generates ceramide much faster than the *de novo* synthesis. Thus, it is of striking importance for rapid signaling pathways. Here, S1P is dephosphorylated via sphingosine kinase and an acyl-CoA is bounded to the formed sphingosine by the action of ceramide synthase forming a ceramide molecule[86]. The third route for ceramide synthesis is the SM cycle, where SM is catabolized by sphingomyelinases (SMases) yielding a ceramide and phosphocholine. There are three different types of SMases according to their pH-dependent optimal activity: acid, neutral and alkaline SMases[87]. Besides, glycosphingolipids can also be hydrolyzed by glycosidases yielding ceramide, although this is not a very important pathway in animal tissues.

Then, the released ceramides are further catabolized by ceramidases, yielding a sphingosine base and a free FA. Markedly, there are five different ceramidases, with different subcellular localizations and FA specificity, thereby affecting different cellular and signaling events. Some of the sphingoid bases generated in this pathway exit the lysosomes and are reutilized for ceramide synthesis via CerS. Likewise, the released sphingosine can be phosphorylated by sphingosine kinases to form sphingosine-1-phosphate that can be hydrolyzed by lyases yielding PE and fatty aldehyde. It is estimated that this pathway accounts for the synthesis of the 50-90% of S1P[82,85,86].

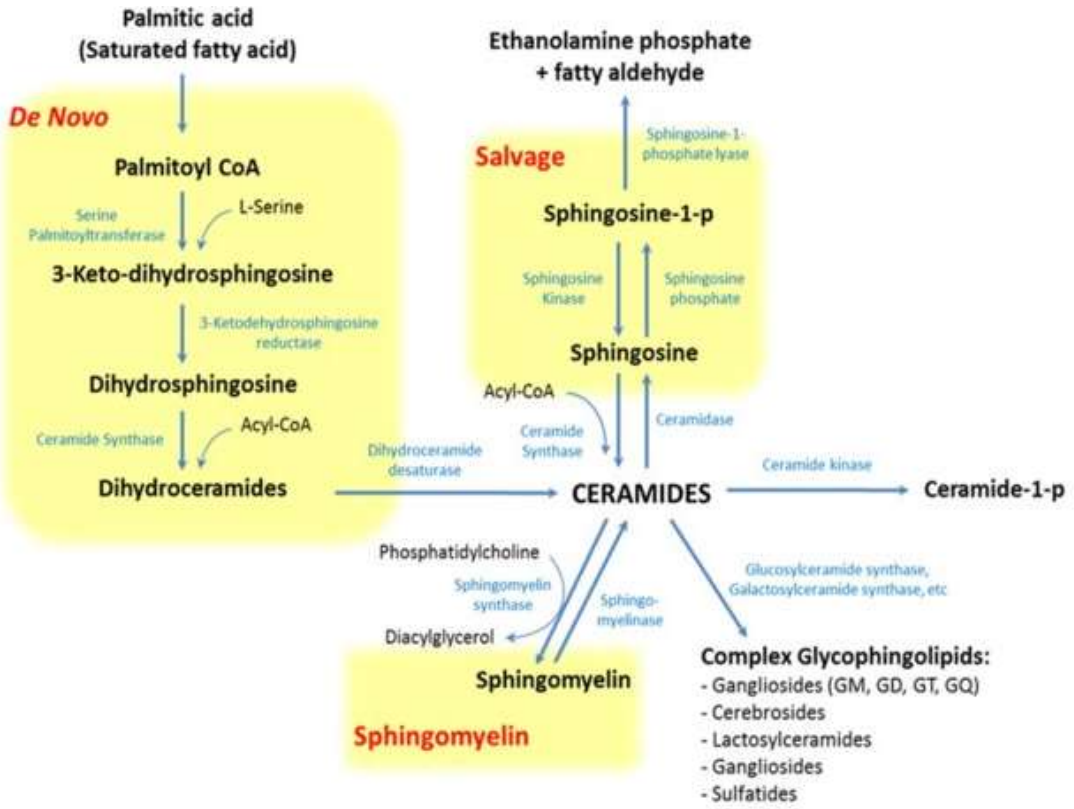


Figure 16. Representation of the metabolic pathways of sphingolipid metabolism[86].

- Sphingomyelin (SM) is the most abundant SPL, and it is ubiquitously distributed in cell membranes, especially in the outer side. It is formed by the addition of a phosphocholine to the position 1 of the sphingoid base of a ceramide. Normally, the base is a sphingosine, and the side chains are long FAs either saturated or monounsaturated odd-numbered chains[87].

  The synthesis of SM takes place in the Golgi apparatus and plasma membrane **(Fig. 16)**, by the linkage of a ceramide and a phosphocholine donated from a PC to a ceramide, and releasing a DAG molecule and a sphingomyelin. This reaction is catalyzed by sphingomyelin synthase (SMS). Additionally, they can also be synthetized by the acylation of a lyso-sphingomyelin. When the biosynthesis takes place in the Golgi apparatus, the ceramide synthetized in the endoplasmic reticulum is transported to Golgi by CERT in an ATP-consuming process. Then, much of the SM produced is transported to the plasma membrane by a vesicular transport mechanism. On the other hand, they are hydrolyzed by SMases releasing phosphocholine and ceramide[81].

  SMs are more abundant in Golgi and plasma membranes, presenting a lower concentration in mitochondria. They are usually located close to cholesterol forming the lipid rafts of the membranes. In fact, several evidences suggest that the metabolisms of these two molecules are connected, and SM levels may control the distribution of cholesterol within the cells. Moreover, SMs control the formation and function of ion channels. Interestingly, they inhibit the activity of Phospholipase A_2, an important enzyme in eicosanoid production and phospholipid remodelling. SM is the most abundant sphingolipid in the nucleus, and participates in several process there. It is significant for the chromatin assembly and dynamics, and it is an important component of the nucleus matrix[82].

- Glycosphingolipids are divided in two groups: Cerebrosides, and glangliosides. Cerebrosides in particular are monoglycosylceramides that are formed by the addition of a carbohydrate molecule to a ceramide. Although they can have different sugars in their structure, glucose (glucosylceramide –GlcCer) and galactose (galactosylceramide – GalCer) are the most common. They have an important structural role in the membranes, especially in the brain. Like SMs, they are usually accumulated in the outer leaflet of plasma membrane together with cholesterol forming lipid rafts, where they bind to enzymes and receptors. They can also be intermediaries of complex glycosphingolipids. Glucosylceramide, although is found in different tissues such as spleen, erythrocytes and nervous tissue, is a major constituent of the skin, and it is required for axonal growth especially in the brain. Furthermore, it has been demonstrated that they are essential for intracellular membrane transport, cell proliferation and survival. On the other hand, galactosylceramides are mainly present in nervous tissue, having an essential role in oligodendrocyte differentiation and myelin structure formation, stability and function. In addition, they have been found to be potent activators of the immune system[88,89]. At the cellular level, cerebrosides are important for cell adhesion in melanoma cells, therefore participating in cell growth and differentiation[89].
  One to five molecules of sialic acid attached to a lactosylceramide form gangliosides. Lactosylceramides are molecules that contain a galactose and a glucose linked to a ceramide. Like cerebrosides, gangliosides are accumulated in the outer leaflet of the plasma membrane forming rafts, and are also important components of the nuclear membrane[90].

Sterol lipids are a family of polycyclic compounds, being cholesterol the most abundant member. Cholesterol is a rigid planar four-ring molecule with a carbon side-chain. It is ubiquitously distributed among all the tissues, and it accumulates mainly in the plasma membrane (30-50% of the lipids in the bilayer). It can be present in a free form, amphipathic form or esterified to long-chain FA forming cholesterol esters. Cholesterol plays a fundamental role in cells, as it is an important structural component of cell membranes, influencing the fluidity of the bilayers as it intercalates between the phospholipids. Moreover, it is the precursor of all steroid hormones and other important metabolites such as Vitamin D and bile acids. Indeed, it is vital for cell signaling, transport, morphogenesis, lipid absorption and digestion, and nerve conduction, among others[91]. Cholesterol esters on their side, are completely hydrophobic molecules and instead of accumulating in the membranes, they are present in the lipid droplets storing cholesterol.

The mevalonate pathway drives the biosynthesis of sterol isoprenoids, such as cholesterol, steroid hormones and bile acids, and nonsterol isoprenoids. This is a very complex process, as at least 30 enzymatic reactions take place in this network. First, the synthesis of the intermediary mevalonate is achieved, which takes place in two consecutive enzymatic reactions, by the union of acetyl-CoA and acetoacetyl-CoA and the action of HMG-CoA synthase, and the subsequent reduction of the previously formed HMG-CoA by HMG-CoA reductase (HMGR)[92]. The latter is the rate-limiting step in mevalonate pathway. If there is low presence of sterol isoprenoids in the cell, the sterol regulatory element binding protein (SREBP) transcription factors are activated and trigger the transcription of HMGR gene. Besides HMGR, SREBPs activate the transcription

of some of the enzymes of the mevalonate pathway[93]. Moreover, SREBPs have also been linked to the increased expression of phospholipid synthesis enzymes[94].

Next, mevalonic acid undergoes two consecutive phosphorylations, yielding two isoprenes. These are consecutively condensed to give squalene. Then, squalene undergoes several enzymatic reactions to finally produce cholesterol.

Other lipid classes that are not studied in this thesis are prenol lipids, saccharolipids and polyketides.

Prenol lipids are synthetized by the subsequent condensation of the five-carbon precursors isopentenyl diphosphate and dimethylallyl diphosphate that are produced in the mevalonate pathway. They contain an isoprenoid tail linked to quinonoid core[83]. Carotenoids are important components of this lipid class, as they are simple isoprenoids that act as antioxidants and precursors of vitamin A. Moreover, vitamin E and vitamin K are other significant lipids within this subclass.

Saccharolipids are formed by a direct link between a FA and a sugar molecule. They are mainly found in bacteria and plants[83].

Polyketides are synthetized after the polymerization of acetyl and propionyl subunits. A large number of the members of this lipid subclass are secondary metabolites and animal products. Among them, there are several anti-microbial, anti-parasitic and anti-cancer agents[59].

2.1.2. Lipid nomenclature

In this work, the shorthand notation has been used. This approach provides a standard and practical nomenclature that is based on LIPID MAPS terminology. For this, every lipid class is provided with an abbreviation that defines its backbone, the head group, and the presence of sugar moieties. In addition, the information about the structure is provided by the description of the acyl chains, which are related indicating the carbon chain length and the number of insaturations[83,95]. Therefore, every lipid species is named by the abbreviation of its lipid subclass followed in parentheses with the number of carbons and the number of insaturations whithin the acyl-chain, separated by a colon. However, in this nomenclature, the type (double or triple bound), position and geometry of the insaturations are missed. For example, a 20-carbon long fatty acid with three insaturations would be denominated as FA(20:3).

Furthermore, if the lipid is a glycerophospholipid with two FA chains, first, the FA esterified in carbon 1 (sn-1) is named and then the FA esterified in carbon 2 (sn-2), separated by a slash. Thus, a glycerol backbone with FA(16:0) esterified in carbon one, FA(20:4) in carbon two and head group of phosphocholine in carbon three would be named as PC(16:0/20:4). However, sometimes it is not possible to define the two FA chains, and the lipids are named as the total sum of carbons and insaturations of both acyl-chains. In this case, it would be noted as PC(36:4).

In the case that the glycerophospholipid has lost one of its FA, the term lyso is used. This is for example LPE(18:0), that stands for lyso-phosphatidylethanolamine with an esterified 18:0 FA. Using this technology it is not possible to reveal in which carbon of the glycerol backbone is esterified the FA.

Besides lipids with esterified FAs , there are ether lipids, which can have the fatty-alcohol linked through either an O-alkyl bond documented as O before the number of carbons and insaturations of the chain, or O-alk-1-enyl bond written as P[95]. For instance, a lipid molecule with

an ethanolamine head group, a fatty-alcohol derived from FA(18:0) linked with an alkenyl bond and a FA(20:4) esterified, it would be named as PE(P-18:0/20:4).

Sphingolipids for their part can be d- or t- in sn-2 depending on the hydroxyl groups present in the sphingoid base: d- for di, and t- for tri. Then, it comes the number of carbons:insaturations of the sphingoid base, followed by a slash and the carbons:insaturations of the FA bound to the sphingoid base[95]. Hence, a sphingomyelin molecule with a di-hydroxyl 18:1 sphingoid base, and with a FA(18:2) linked to its structure, it would be denominated as SM(d18:1/18:2).

Table 4. Abbreviations of the lipid classes detected in this work.

Lipid Class	Abbreviation
Free Fatty Acid	FFA
Glycerolipids	GL
Diglycerides	DG
Triglycerides	TG
Glycerophospholipids	GPL
Phosphatidic Acid	PA
Phosphatidylcholine	PC
Lysophosphatidylcholine	LPC
Phosphatidylcholine ether	PC(P/O)
Phosphatidylserine	PS
Phosphatidylethanolamine	PE
Lysophosphatidylethanolamine	LPE
Phosphatidylethanolamine ether	PE(P/O)
Phosphatidylinositol	PI
Phosphatidylglycerol	PG
Sphingolipids	SPL
Ceramides	Cer
Sphingomyelin	SM
Hexosylceramide	HexCer
Sterols	
Cholesterol esters	CE

2.2. Lipidomic analyses

The heterogeneous nature of melanoma tumors hampers to find a completely accurate biomarker and treatment option for melanoma. Furthermore, the variability of the carcinogenic process is such that even tumors with the same histologic features, share different genetics, proteomics, metabolomics and epigenetics, thus presenting a distinct proliferative and metastatic profile. A better understanding of the molecular changes within neoplasms is of vital importance. In line with this, the study of the lipid content of cells and their metabolism has aroused great interest in recent decades.

Lipidomics was first defined in 2003 as an analytical approach that aims to characterize and quantify the lipid content of a sample, as well as identify the interactions of these molecules with other biomolecules such as other lipids, proteins or genetic material. From then on, lipidomic analyses have evolved as a promising field in cancer research and biomarker discovery. To date, these approaches are frequently used to detect and classify tumor cell lines and tissues, in order to eventually differentiate normal and tumor tissues[96–99].

Owing to the physical and chemical diversity of lipids, there is no single analytical process that studies the entire lipidome of a cell or sample in a single attempt. However, several analytical procedures have been developed recently that allow the identification of a broad spectrum of lipids. The rapid evolvement of this field has been tightly connected to the great advances made in mass spectrometry techniques. Although these studies can be performed using NMR (nuclear magnetic resonance), Raman spectroscopy and other spectroscopic approaches, most lipidomic methodologies are mainly based on mass spectrometry methods (MS), which can be performed either directly on the samples or combined with a previous liquid chromatography. A common workflow in lipidomic analyses comprises lipid extraction from the biological sample, possible chromatographic separation, soft ionization and MS-based analysis, and data processing.

In this work, we have used two different lipidomic methodologies, that are two of the most used mass spectrometry strategies for lipidomic studies: UHPLC coupled to ESI-MS/MS tandem methodology, and MALDI-MS methodology[96–99].

2.2.1. UHPLC-ESI-MS/MS methodology

In this approach, lipid extracts of the cell lines studied were required, so, the first step was to optimize the best extraction conditions for our samples. Before choosing the most suitable extraction method, the extraction percentage of the lipids in the sample must be assessed performing a recuperation experiment comparing different methodologies. For this, a known quantity of different lipid-standards that represent the lipid families present in the study samples are added to the samples before performing the extraction. In addition, a control condition is needed. Here, an intact sample is extracted and the lipid-standards are added after the extraction. In this way, the intensity obtained for the standards would be representative of the total amount of standard added. Then, the intensity achieved for the standards in the samples is compared with the intensity of the standards of the control condition. In this way, the intensity difference represents the percentage of sample that is recovered from the extraction, this is, the recovery rate. The the Bligh & Dyer method shows overall better recovery percentages than the protein precipitation method with isopropanol **(Table 5)**. Therefore, Bligh & Dyer method was chosen as the gold standard method for the lipid extraction of the small biological samples used in this work.

Table 5. Recovery rates (%) of the different standards performing the lipid extraction of a sample using Bligh & Dyer method and protein precipitation with isopropanol.

Lipid class	Lipid Standard	Recovery rate (%)	
		Bligh & Dyer	Isopropanol
Glycerolipids	TG(14:0-16:1-14:0)	97	84
	TG(16:0-18:0-16:0)	94	66
	TG(15:0-18:1-15:0)	93	88
	TG(17:0-17:1-17:0)	100	88
Glycerophospholipids	PC(17:0/14:1)	102	95
	LPC(17:1)	82	91
	PI(17:0/14:1)	46	86
	PG(17:0/14:1)	70	95
Sphingolipids	SM(d18:1/12:0)	96	87
	Cer(d18:1/25:0)	105	116
	Cer(d18:1/12:0)	91	91
	GlcCer(d18:1/12:0)	94	93
	LacCer(d18:1/12:0)	89	103

In the Bligh & Dyer method, a two-phase separation is achieved, using chloroform and methanol-water solvents. In the non-polar solvent, this is chloroform, the hydrophobic lipids are dissolved and thereby extracted, while the polar components are concentrated in the polar methanol solvent in order to break the hydrogen bonds or electrostatic forces that bind them to the proteins of the membrane. In addition, the alcohol-solvent generates the inactivation of many phosphatidases and lipases, preventing the enzymatic degradation of the lipids of the sample[100]. Therefore, this procedure enables the separation of the lipids that contain glycerol, steroids, ceramides and sphingomyelins in the chloroform phase, whereas the polar lipids, such as phosphorylated PIs, complex glycolipids, specially gangliosides, and non-lipid components such as sugars are retained in the aqueous phase[101].

As pointed out in the previous section, the cellular lipidome is a very complex mixture of lipids, considering that within each lipid class there is lipid species galore. Thus, chromatographic pre-separation of the lipid families is often used, since the correct identification of isobaric molecules can be challenging. Therefore, different chromatographic approaches coupled to MS are widely used in lipidomic studies. [96–99].

The next step in this approach was to separate the lipid classes using chromatographic techniques. Concretely, ultra-high pressure liquid chromatography (UHPLC) was used, as it is a very advantageous technique for the separation of amphipathic lipids based on their physicochemical properties. Indeed, when using reversed-phase UHPLC, lipids of the same class can be separated based on their lipophilicity, which is determined by their acyl chains' length and number of double bounds. For instance, the elution time is faster for lipids with short acyl-chains and polyunsaturated acyl structures[102]. Moreover, UHPLC strategy is very convenient for separating phospholipids in the different subclasses[96]. Since the chromatograph is coupled to the spectrometer, as soon as the components of the mixture are separated according to their retention time, they are introduced in the spectrometer and identified via MS-based analyses.

Mass spectrometry techniques allow the identification of different charged molecules (ions) according to their mass-to-charge ratio (m/z), providing both structural and quantitative information of the analytes (ions). The essential parts of a spectrometer are the ion source, the

mass analyzer, the detector and the data system[103]. The extracted lipids, with or without previous separation by UHPLC approaches, enter the ion source where they are ionized, thereby acquire positive or negative charge. Afterwards, the ions pass through the sampling cone and reach the analyzer. The aim of the analyzer is to solve the generated ions according to their mass-to-charge relationship. For example, when quadrupole-time-of-flight (Q-TOF) analyzer is used, the quadrupole selects the ions and they are sent to the collision cell for argon fragmentation. These molecules reach the time-of-flight analyzer, which applies an electric field to accelerate all the ions to the same potential, and the time that takes each of the ions to reach the detector, which depends on its mass, is measured. Ions with a higher mass arrive later at the detector. Eventually, the detector records the information of the mass of the ions and their abundance in the mixture, which is then sent to the data system where it is graphically represented as mass spectra. Each peak of the graph represents a specific mass present in the mixture, and the amplitude of each peak stands for the relative abundancy of that ion.

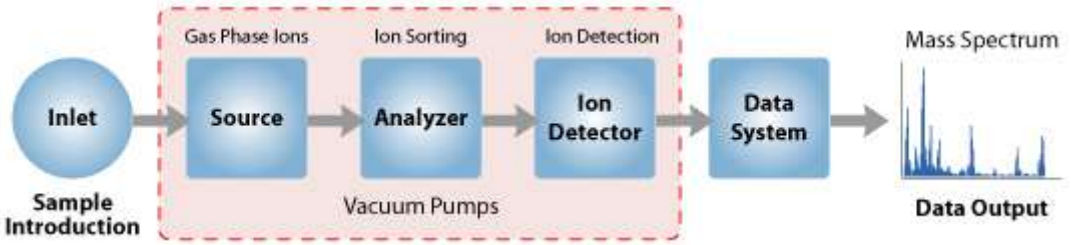


Figure 17. Schematic representation of the different components of a mass spectrometer[103].

The great progress made in the lipid research field has been largely due to the advances achieved in the soft ionization MS techniques. These include ESI (electrospray ionization) and MALDI (matrix-assisted laser desorption and ionization). ESI uses electrical energy to transfer the ions present in the extraction solvent or mobile phase of UHPLC to a gaseous phase such as an aerosol. In order to evaporate the solvent and obtain the aerosol, the solution is pulverized through a quartz capillary with a high potential, together with a stream of hot nitrogen that help to evaporate the solvent from the charged droplets. Eventually, the solvent-free sample ions pass through the sampling cone and arrive at the analyzer and the detector. The UHPLC-ESI-MS approaches increase greatly the sensitivity and accuracy for detecting low abundant lipids. Additional information about the structure of the molecule can be provided using tandem MS/MS techniques[104]. Here, two different analyzers are used; first the precursor ion is revealed, and then this molecule is fragmented in a collision cell, and thereby detected by a second mass analyzer. In this way, the structure of the precursor molecule is more efficiently identified[96,98]. It is important to highlight that ESI is the preferential ionization method for studying lipids in solution[105].

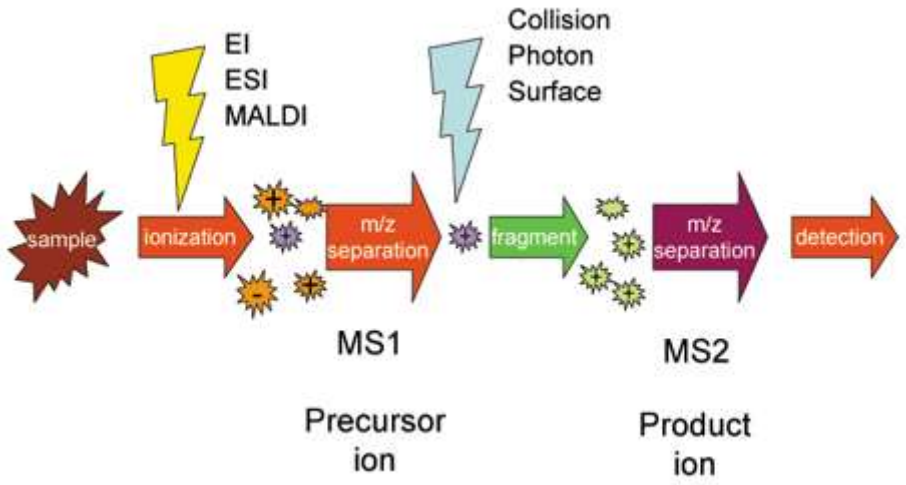


Figure 18. Workflow of tandem MS/MS strategy[104].

2.2.2. MALDI-MS methodology

The second analytical procedure employed was based on lipidomic analysis of functional cell membranes using MALDI-MS methodology. For this, the first step was to extract the cell membranes of the cell lines studied and immobilize them fabricating a functional cell membrane microarray, using a technology property of IMG Pharma Company. Then, for the lipidomic study, MALDI-MS strategy was performed.

MALDI-MS technique is frequently used for biomarker discovery. It is a laser-based soft-ionization technique, where a thin layer of low-molecular-weight organic matrix is applied on top of the sample. This matrix absorbs the laser radiation, and emits molecular ions and fragmented ions. One of the critical steps in this analytical process is the sample preparation. Indeed, matrix selection and deposition is strikingly important. It has been demonstrated that the best matrix deposition method includes matrix sublimation to ensure a regular distribution of it along the sample, and the use of matrixes that form small crystals. MBT (2-mercaptobenzothiazole) and DAN (2,5-diaminonaphtalene) for positive and negative ion detection, respectively, have shown great specificity and a good identification of the different lipid species[106]. This analytical approach is suitable for polar lipids including phospholipids and sphingolipids, and some non-polar lipids such as DGs and TGs[98,107].

2.3. Cancer metabolism

Cancer cells undergo molecular changes that support the acquisition and maintenance of the carcinogenic phenotype, what gives them the advantage to survive, proliferate and grow in the stressful conditions generated by the tumorigenesis process[108]. In order to support the anabolic and energetic needs of tumor cells, the metabolic program of the cells is accurately rewired, what is directly regulated by translational and posttranslational modifications.

In brief, under normal conditions in healthy cells, the absorbed glucose is mainly transformed into pyruvate in the cytosol and metabolized by the oxidative phosphorylation (OXPHOS) in the mitochondria, producing 36-38 ATP molecules per molecule of glucose[109].

Conversely, malignant cells use the hypoxic response used by normal tissues, regardless of the availability of oxygen **(Fig. 19)**. This is defined as the Warburg effect, which is the first metabolic alteration detected in cancer cells and was described in the first half of the 20th century by Otto Warburg. Significantly, it states that the main route of glucose metabolism in cancer cells moves from oxidative phosphorylation to lactate fermentation, decreasing significantly the production of ATP molecules[110–112]. Melanoma in particular, has been defined as a very glycolytic cancer, as 60-80% of the absorbed glucose is converted into lactate in normoxia, increasing to 90% or more in hypoxia situations[113]. The physiological consequences of the metabolic plasticity observed in cancer cells are bioenergetics alterations and augmented synthesis of biomolecules, since the carbons derived from glucose are not used for obtaining energy but as biosynthetic building blocks for macromolecules such as lipids, amino acids and nucleotides.

Until recently, it was thought that cancer cells had impaired mitochondria, so the electron transport chain could not generate ATP, believing that this was the reason why these cells use aerobic glycolysis. However, it has been shown that mitochondria operate properly and glutamine is the principal carbon supplier of tricarboxylic acid cycle (TCA), instead of glucose[110–112]. In this way, glutamine is considered an important source of energy for proliferating cells. Additionally, glutamine also supplies carbons for the synthesis of lipids and the amino-nitrogens needed for the synthesis of amino-acids, nucleotides and lipids[110,114,115].

Another source of energy for cancer cells is the oxidation of fatty acids. Here, these molecules are shortened by a series of catabolic reactions, generating acetyl-CoA that is used to replenish the TCA cycle, generating twice as much ATP as carbohydrates, and NADPH. The use of this energy source is emphasized when the availability of nutrient and oxygen is scarce, and the highest levels of use of fatty acid oxidation have been related to aggressive tumors[115,116].

Aside ATP, NADPH is also needed to support the increased anabolic pathways in cancer cells. Moreover, NADPH also protects cancer cells from oxidative stress. In addition to the mitochondrial fatty acid oxidation (FAO), the pentose phosphate pathway (PPP) also provides NADPH after the oxidation of glucose[117].

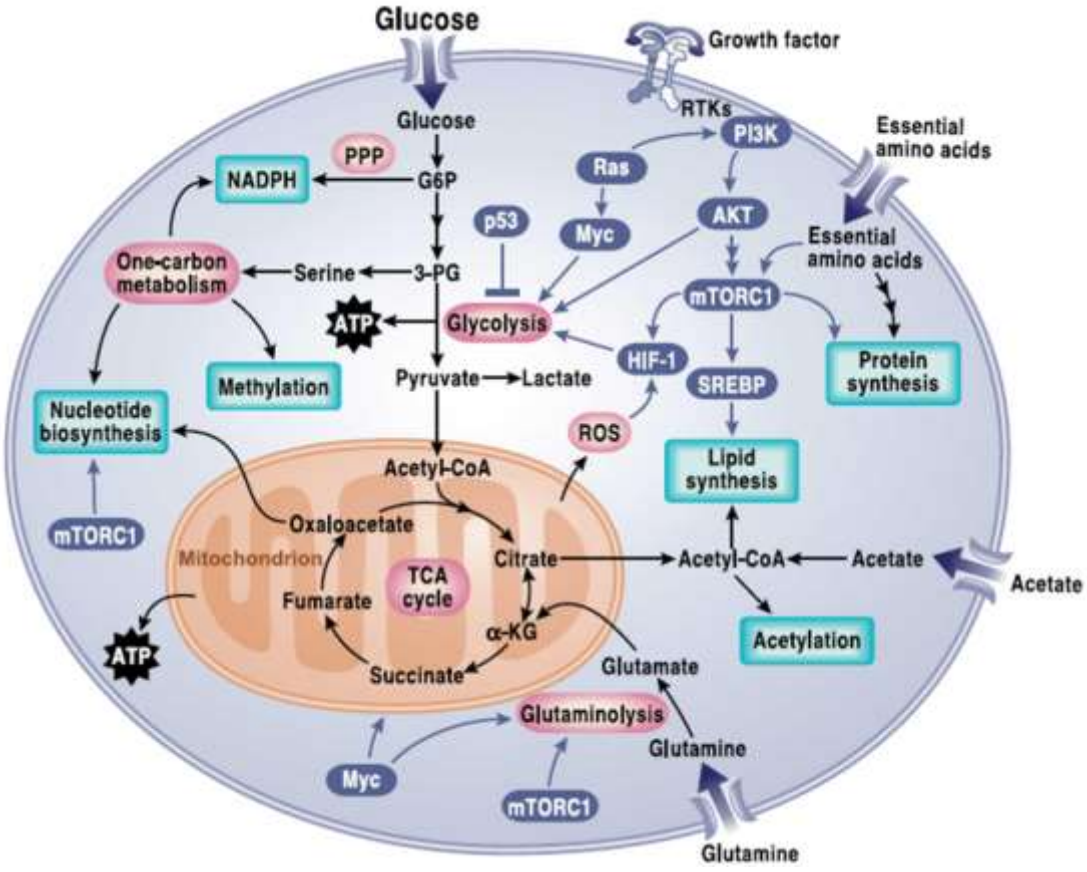


Figure 19. Schematic representation of the signaling pathways that regulate metabolic rewiring in cancer cells[108]. The aberrant activation of different metabolic pathways in cancer cells increases the biosynthesis of lipids, nucleotides, and proteins. In addition to the increase of glucose absorption and glycolytic flux. Abbreviations: PPP –pentose phosphate pathway; G6P –glucose-6-phosphate; 3-PG -3-phosphoglycerate; ATP –adenosine triphosphate; α-KG –α-ketoglutarate; RTK –receptor tyrosine kinase.

2.3.1. Lipid metabolism and cancer

The alteration of lipid metabolism has gained attention in recent years. Compelling evidence suggests that both *de novo* biosynthesis and oxidation of these macromolecules are increased in cancer. The rewiring of lipid metabolism results in disrupted energy production, distinct cell signaling pathways, structural alterations in cell membranes, and aberrant gene expression and protein distribution, affecting significant cellular functions, such as growth, proliferation, apoptosis, autophagy, differentiation, and resistance to drug and chemotherapy, among others[49].

Malignant cells show great need for lipids including cholesterol, so both the synthesis and the uptake of these molecules are increased in cancer, storing the excess in lipid droplets (LD) within the cells, which is considered a cancer trait. Melanoma particularly shows a great necessity of FAs for its progression and metastasis, using them for energy extraction by FAO or membrane phospholipid synthesis, among other functions. Aggressive cells show higher levels of expression of the genes involved in lipid biosynthesis, catabolism and intracellular storage of lipids. Besides, it has been reported that lipid metabolism participates not only in the first steps of the metastatic process, but also in peripheral dissemination and seeding. In fact, both the

pharmacological and molecular blockade of this process with siRNAs has led to cell apoptosis and a decreased primary tumors volume[114,118].

The metabolic rewiring of cancer cells drives the aberrant activity of SREBP transcription factor, the master regulator of fatty acid, phospholipid, triglyceride and cholesterol metabolism. SREBP controls lipid metabolism at transcriptional, translational and post-translational levels. This transcription factor promotes the upregulation of the expression and activity of several lipogenic and lipolytic enzymes. For instance, FAS (fatty acid synthase), ACC (acetyl-CoA carboxylase), ACLY (ATP citrate lyase), and SCD (stearoyl-CoA desaturase) enzymes upregulation is a common phenotype observed in a wide range of cancers, including melanoma. As it is represented in the **Figure 20**, these enzymes participate directly in the *de novo* biosynthesis of FAs, and in the modification of the new FA molecule by elongating its carbon chain and introducing insaturations[94]. It is noteworthy that the pharmacological inhibition of these enzymes reduces the invasion, migration and survival of cancer cells *in vitro*[114,119].

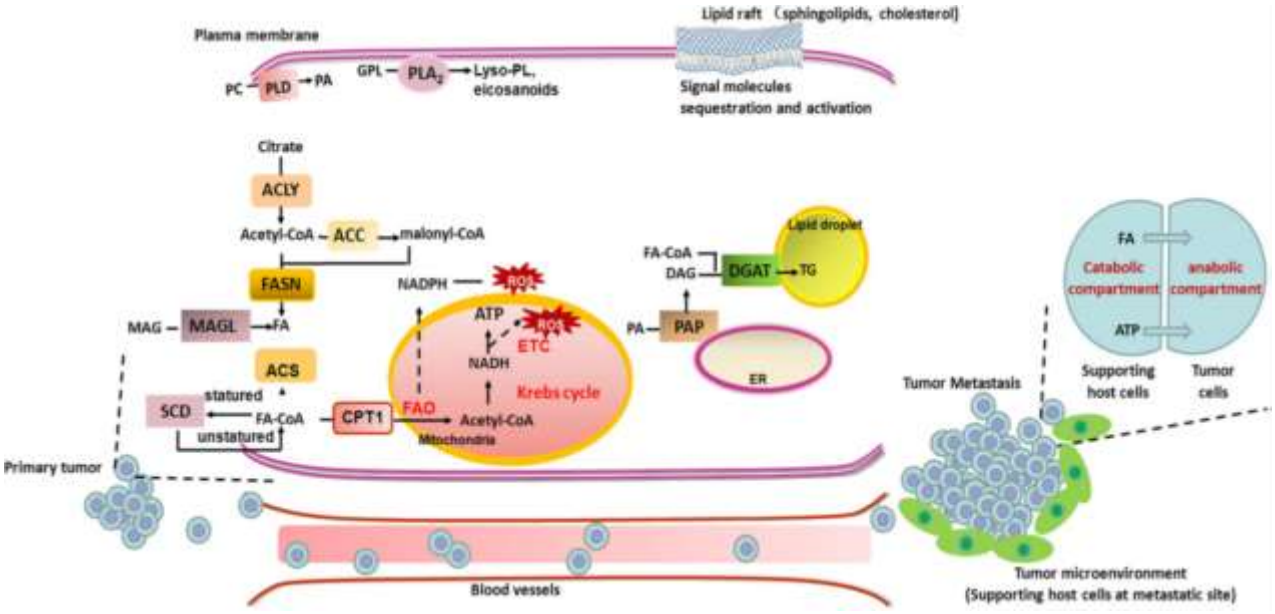


Figure 20. Schematic representation of the lipid metabolism rewiring in cancer cells[120].

Although FA synthesis and oxidation are a priori incompatible processes, they coexist in cancer cells taking advantage one from the other. FA oxidation produces acetyl-CoA accumulations in the cells, which can be used for FA synthesis initiation. Furthermore, the ATP and NADPH generated in the oxidation are profited for anabolic purposes. The cells eliminate toxic lipids hydrolyzing them and, at the same time, FA synthesis generates the concrete lipid species required for the malignant transformation of the cells[120].

The metabolism of the different phospholipid subclasses is also altered in cancer, as the biosynthesis of phospholipids is stimulated, thereby increasing their content in tumor cells[121,122]. Overwhelming evidences suggest that the enzymes that participate in the Kennedy pathway are overexpressed in tumor tissues. Concretely GPAT2, part of the GPAT family of enzymes that catalyze the first reaction of this stepwise process is overexpressed in some cancer tissues[77]. Choline kinase (CK), which is responsible for the first step of PC biosynthesis, has an increased

presence and activity in cancer cells, including breast, ovarian, lung, colon, prostate, endometrial, pancreatic cancer and melanoma[123]. It participates in tumor development, progression and metastatic dissemination in several cancers[122]. In line with this, numerous CK inhibitors are under study for clinical development, after showing great efficacy *in vitro* and *in vivo* in different cancer cell lines[55,124]. In order to maintain a high PC biosynthetic rate, the absorption of free choline by cancer cells is also stimulated. The proteins that participate in the transportation of choline inside the cells appear upregulated in different cancer cells, including melanoma. In fact, the inhibition of these proteins through different mechanisms has reduced tumor cell proliferation, increased apoptosis and blocked tumor progression[123]. Besides, the catabolism of PCs is also enhanced in tumor cells. This is strikingly important in tumor progression, and is performed by phospholipases A, C and D, yielding very important second messengers such as, DAG, PA, LPA, FFAs, Lyso-PC, free choline and phosphocholine[121].

In spite of lacking evidences of the exact role of the plasmalogens in cancer, a large body of work demonstrates the overexpression of various enzymes that take part in the biosynthesis of ether lipids in cancer tissues. Moreover, it has been demonstrated that ADAPS is overexpressed in several cancers, what is turned into higher levels of alkyl ether lipids, favoring cancer progression[74]. Indeed, the knockdown and inactivation of this enzyme has been associated to impaired cancer progression, whereas its overexpression and thus increased ether lipids content has been associated to increased tumor cell growth, motility and survival[70,125].

FAO not only occurs in mitochondria, but also in the peroxisomes. Although the oxidative mechanism is identical in both organelles, the enzymes involved in each organelle are different as they metabolize different FAs. Indeed, branched and very long-chain fatty acids (>26 carbons) can only be hydrolyzed in the peroxisomes, where the first oxidative reactions take place, breaking the long-chains into smaller ones, which are then shuttled to the mitochondria to continue the oxidation[74,75].

In addition, the specific lyso-phospholipid transferases (LPATs) and PLA_2 that participate in the Lands' cycle are overexpressed in several cancers, such as, liver, colon, prostate, lung, gastric and breast cancers[77]. The increased production of important signaling molecules such as lyso-PLs and free fatty acids, is turned to enhanced proliferation, migration and metastatic capacity in several cell lines[96].

With regard to sphingolipids, a common scenario in several cancers is characterized by the increased activities of glucosylceramide synthase (GCS), SM synthase (SMS), ceramide kinase (CERK), acid ceramidase (aCDase) and/or sphingosine kinase (SK), which increases the expression of pro-carcinogenic SPLs[84]. In general, the accumulation of ceramides in the cells triggers apoptosis, although it has been demonstrated recently that this is ceramide-type specific. For instance, cancer cells present low levels of C18 ceramides due to the decreased expression of CerS1, whereas the increased expression of CerS2 is translated into accumulation of long-chain ceramides (22-24 carbons-length) in cancer cells[84,85]. Particularly in melanoma, low expression of CerS6 was correlated with poor prognosis. To gain insight into the importance of this enzyme in melanoma progression, its expression was silenced and the cell lines showed increased progression and invasion[126]. Moreover, the *in vitro* overexpression of CerS5/6 that induces C16 ceramide production and accumulation, enhanced apoptosis in cancer cells. Interestingly, upon satisfactory chemotherapeutic treatments, long-chain ceramides tend to accumulate and CerS4, 5 and 6 are found overexpressed[85].

On the other hand, melanoma among other cancers show high levels of acid ceramidases. Compelling evidence suggest that lysosomal aCDase participates in the transition from proliferative to invasive phenotype in melanoma cells. In line with this, higher expression of aCDase in melanoma cells has been related to a proliferative state, whereas lower expression has been correlated to enhanced motility and invasive capacity[127]. Furthermore, aCDase overexpression has been proven to confer resistance to chemotherapeutic agents, highlighting the importance of these enzymes in the carcinogenic process[128].

According to the role of SM in cancer, SM synthase (SMS) downregulation has been correlated to poor outcome in melanoma, thereby decreasing SM levels in primary and metastatic melanoma[129]. Interestingly, acid SMases have been proven pivotal in the metastatic process of melanoma[84].

The mevalonate pathway is another lipogenic route that has increased activity in cancer. As explained previously, several evidences suggest that many cancer types present high activity of SREBP transcription factors that promote the expression of the enzymes of the mevalonate pathway. When the pathway is activated, the cells synthesize cholesterol that is accumulated in high levels in different tumor tissues, such as breast cancer[130]. Cholesterol esters on their side, are synthetized by acyl-CoA:cholesterol acyl transferase (ACAT), which is found overexpressed and correlates with tumor grade in different cancers such as clear cell renal carcinoma[131]. Interestingly, statins, that lower plasma cholesterol levels and are so largely used in clinic, have been proven to reduce cancer cell proliferation, induce apoptosis and make cancer cells more sensitive to chemotherapeutic agents *in vitro*. In fact, the administration of statins together with chemotherapeutic agents has shown greater efficacy than drugs alone. However, these effects are still under study since they appear to be very specific to the cancer type[132].

In summary, it has largely been demonstrated that cancer cell metabolism, and concretely lipid metabolism, are altered in cancer. This metabolic rewiring supports the acquisition of the malignant traits that cancer cells possess. Therefore, the inhibition of the key enzymes that participate in lipid metabolism arises as a promising treatment option for cancer patients.

3. Phospholipase D and cancer

Phospholipases form a ubiquitous family of enzymes that participate in the catabolism of glycerol-based phospholipids. The members of this family can be divided in four major classes, each of which hydrolyses different phospholipid molecules in a specific position **(Fig. 21)**. They are pivotal both in physiological and pathological conditions, since they are necessary for the remodeling and homeostasis of the plasma membrane. Indeed, they have been largely related to carcinogenesis and other diseases, as the released molecules are bioactive lipids that regulate diverse intracellular and intercellular signaling pathways, which affect cell proliferation, survival, migration, vesicle trafficking and cell death, among others. Moreover, many of the different members of this family have been found to be overexpressed in several cancers[133].

Enzymatic action of the diverse phospholipases:

- PLA: Phospholipase A_1 and A_2 can cleave the ester bond of the GPL at sn-1 or -2 position respectively, releasing a free fatty acid and a lysophospholipid.
- PLB: presents both PLA_1 and PLA_2 activities.
- PLC: cleaves the bond between the phosphate and the glycerol at sn-3, producing a DAG and a phosphorylated head group.
- PLD: cleaves the bond between the phosphate and the polar head group of the glycero-PL, releasing a PA and a free polar head group.

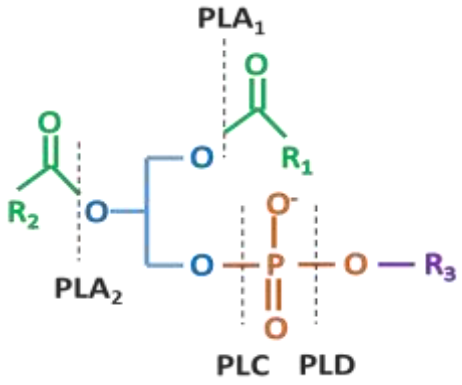


Figure 21. Cleavage sites of the different phospholipases.

In particular, the PLD subclass is composed of six different isoforms in mammal cells. So far, PLD1 and PLD2 are the best-studied members. Although both show the same catalytic activity, that is, they hydrolyze preferentially a PC yielding PA and free choline, their tissue and subcellular location is different. PLD1 is found mainly in the inner leaflet of different perinuclear membranes, such as secretory granules, endosomes, lysosomes and Golgi apparatus. Once activated, it is transported to the plasma membrane. There, it exerts its hydrolytic activity influencing membrane trafficking, mitosis and signal transduction. This isoform has been linked to cancer, thrombotic disease and autoimmunity. Conversely, PLD2 is found primarily in the plasma membrane and has been related to strong catalytic activity. Indeed, the lipase activity exerted by PLD2 has been linked to cytoskeleton reorganization, regulated secretion and cell cycle control. Similar to PLD1, PLD2 has also been reported to be related to cancer, as well as hypertension and Alzheimer's disease. Additionally, PLD3, PLD4, PLD5 and PLD6 isoforms are also found in mammal cells. However, not much is known about their functions. It has been shown that PLD3, PLD4 and PLD5 isoforms are anchored in the plasma membrane by a

transmembrane domain. However, PLD6 has been described as a dimeric protein that can be found on the external surface of the mitochondria[133].

PLD1 and PLD2 share a 50% homology among their sequences, although PLD2 is shorter. A common structural feature that they share is a conserved motif of phosphatidyltransferase HKD that is duplicated and encodes the catalytic site of these enzymes. This domain is characterized by the amino acid sequence $HxKx_4Dx_6$, with histidine, lysine and aspartic acid, where x represents any amino acid residue. Moreover, at the amino terminal region of both PLD1 and PLD2 there is a tandem of PX (phox homology) and PH (pleckstrin homology) domains. This tandem participates in the interactions with the lipids of the membranes and controls the subcellular localization of the enzymes. However, these domains do not participate in the catalytic activity of these proteins[134]. With respect to the remaining PLD isoforms, neither PLD3, PLD4, PLD5 nor PLD6 possess PH and PX domains within their sequence. Furthermore, all but PLD6 have two HKD motifs, that has a single HDK domain[135].

PIP_2 (phosphatidylinositol 4,5-biphosphate) is a fundamental cofactor for the catalytic activity of PLDs, hence, a PIP_2 binding region has been found. Bruntz et al. mutated this region, showing that the catalytic activity suffered a significant decrease, while the subcellular location was not affected. In addition, it has been postulated that the role of PIP_2 could be to recruit PLD to a concrete membrane domain and stimulate PC catalysis by facilitating the binding of the substrate to the active site[134].

Despite being the lipase activity the best-known activity of PLDs, these enzymes also show protein-protein interactions. The catalytic activation of these enzymes occurs in two different pathways. On the one hand, lipase activity is driven by phosphorylation through receptor tyrosine kinases (RTKs), such as EGFR or PDGFR, and non-receptor tyrosine kinases including protein kinase C (PKC), Src and janus kinase 3 (JAK3)[135]. In this way, different extracellular mitogenic signals stimulate the activation of PLD: chemokines (IL-8), insulin, growth factors (epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF)), fatty acids, sphingosine-1-phosphate (S1P), among others[136]. On the other hand, the guanine nucleotide exchange factor (GEF) activity is a unique feature of the PLD2 isoform. This is implicit in cell motility, and the regulation of PLD2 activation is carried out through small GTPases such as Rac2, Arf, Rho and Ras, among others. Furthermore, it has been found that PA controls the GEF activity of PLD2, what adds greater sophistication to the regulation of this enzyme[135,137].

Upon activation, the formed PA can be subsequently converted to lyso-PA by PLA_2 or to DAG by PA-phosphohydrolase. Therefore, PLD is a key enzyme in cell signaling, as the molecules produced are important lipid mediators involved in several cellular signaling pathways. In fact, one of the important features of PA is its interaction with some proteins, many of which participate in cellular signaling pathways. For instance, mTOR, Akt and Raf-1 (member of ERK signaling pathway) proteins interact with PA, which implies the influence of PLD on cell proliferation, growth, and survival[133].

Specifically in cancer, in addition to the regulatory transcriptional mechanisms, post-transcriptional mechanisms also contribute to the enhanced expression and activity of these enzymes[138]. Thus, both the expression and the activity of PLD1 and PLD2 are upregulated in several types of neoplasms, including breast, kidney, colon, gastric, brain, thyroid, as well as melanoma[139]. PLD2 in particular, correlates with poor prognosis in colorectal and renal neoplasms[140]. Overexpression of PLD and increased activity affect a wide range of processes

involved in tumor progression and metastasis by different signaling pathways. The increase in PLD activity due to mitogenic signals (EGF, PDGF) and the expression of the oncogenes (Ras, Raf, Src) has been linked to the activation of MAPK, RAS and mTOR[140,141]. Moreover, HIF increases the expression of PLD2[142], and PLD activity contributes to rapamycin resistance and survival of human bladder, breast and lung cancers through mTOR[143,144]. In this regard, PLDs are important to maintain altered cancer metabolism, supporting the biosynthetic and bioenergetic demands of cancer cells. For instance, PLD enzymes increase the uptake of glucose by Glut-4 and the formation of LD. In line with this, PLD interacts with both the tumor and the microenvironment, favoring proliferation and stimulating angiogenesis[142].

One of the effects of PLDs on cancer that stands out over the others is their role in the metastatic process. Both the PA produced by their lipase activity and their protein-protein interactions with different signaling molecules such as Grb2, Rac2, WASP, S6K and JAK3, among others, are key to induce cell motility. However, the expression levels of PLD1 in the metastatic sites do not differ significantly with those of the primary renal tumors. Therefore, it is suggested that although PLD1 also contributes to the metastatic process, PLD2 might play a pivotal role in tumor cells for metastatic dissemination[140]. Moreover, it is postulated that PLD1 exerts its function mainly in the tumor microenvironment, whereas the activity of PLD2 is intrinsic to tumor cells. In line with this, some information can be gleaned from different studies. Once the migratory cells enter the circulatory system, they attach to the platelets in order to protect them and direct them to the secondary tissue. It has been demonstrated that the activity of PLD1 is pivotal for the aggregation of tumor cells to the platelets via integrins. To gain insight into the role of PLD1 in this process, PLD1 knockout mice were intravenously injected with wild type melanoma cells, noticing a 50% decrease in the formation of metastases in these mice. Besides, the deletion of PLD1 in endothelial cells led to a decrease in neovascularization of tumors[145].

On the other hand, PLD2 has been strongly linked to metastatic dissemination. PLD2 activity supports the proliferation and invasiveness of different cancers, such as lymphoma[146] and breast cancer[139]. Moreover, PLD2 has been demonstrated to induce the metastatic process by phosphorylation of FAK, and activation of Akt and mTOR[146]. In fact, PLD2 supports matrix substrate degradation, invadopodia formation, tumor cell migration, invasion and metastatic spread of breast cancer cells. It increases the secretion of matrix metalloproteinases from tumor cells and promotes the rearrangement of the actin cytoskeleton[147], while its inactivation impairs cell proliferation, adhesion, migration and invasion[146]. In breast cancer, overexpression of PLD2 resulted in increased tumor growth, chemoresistance and reduced apoptosis in mice, whereas silencing of PLD2 significantly impaired tumor growth and lung metastases[139].

HYPOTHESIS & OBJECTIVES

1. Hypothesis

Melanoma is the cancer that arises upon the malignant transformation of the melanocytes of different locations, although skin melanoma stands for the most common one. Its incidence has increased steadily during the last decades, and is considered the seventh most diagnosed cancer in Europe. Although it only represents the 4% of all diagnosed skin tumors, it is responsible for 80% of the deaths caused by these cancers[1]. It should be noted that it is predicted that 150,000 new cases will be diagnosed in Spain during 2019[3].

The great heterogeneity observed in melanoma tumors has hindered the identification of truly effective biomarkers or treatments, and mortality rates are still very high, especially for metastatic melanoma. Therefore, it stands to reason that there is great need to identify new biomarkers that can be used as therapeutic targets or help in the early detection, diagnosis and prognosis, as it is evident that the recovery rates are higher the earlier the tumor is detected.

In this regard, cellular metabolism has been established as a cancer trait. It is known that the rewiring of metabolic pathways that these cells undergo supports the malignant phenotype acquired by the tumor cells. Specifically, the implication of lipid metabolism in the carcinogenic process has gain insight in the cancer research field. The metabolism is known to be altered in cancer cells, which induces modifications in the composition and quantity of some lipids. Furthermore, it has been hypothesized that these alterations in the composition and quantity of lipids and their metabolism can lead to the malignant transformation of non-pathological melanocytes to melanoma. Thence, the specific lipid species identified with an aberrant expression in melanoma compared to non-pathological melanocytes could be important potential biomarkers for this disease and help to shed light in their involvement in the development and progression of melanoma. Moreover, the variations in the lipid content go together with the alterations in the expression and activity of the enzymes that participate in the metabolism of these molecules. For instance, phospholipase enzymes participate in the catabolism of the glycerophospholipids, and the different isoforms of this family of enzymes has been largely related to cancer. Indeed, PLD2 has been shown to contribute in the carcinogenic process and metastatic dissemination of different cancers.

2. Objectives

The main objective of this doctoral thesis was to identify new melanoma lipid biomarkers that will aid in the early detection, diagnosis and prognosis of melanoma. For that purpose, different lipidomic approaches were applied. In addition, it was expected that the results obtained in this work would gain insight into the molecular and cellular mechanisms involved in development and progression of melanoma.

For this, the main objectives are itemized in the following specific objectives:

- Analyze the lipidome of healthy melanocytes, nevi melanocytes, primary melanoma and metastatic melanoma cell lines, using lipid extracts obtained from various cell lines.
- Implement an effective new biotechnological tool with translational potential for lipidomic biomarker detection.
- Identify specific lipid species with diagnostic and prognostic value for melanoma.
- Determine the functional implications of PLD2 enzyme in pro-tumorigenic and metastatic activities in melanoma cells.

MATERIALS & METHODS

1. Materials

1.1. Reactants

Table 6. List of the reactants used for the experiments and the supplier.

Product	Supplier
RPMI 1640 Glutamax	Life Technologies
Mc Coy's 5A	
Medium 254	
Human Melanocyte Growth Supplement (HMGS)	
Penicillin (100 UI/mL) – Streptomycin (100 µg/mL)	
TryPLE Select (1x)	
0,05% Trypsin-EDTA (1X)	
Hank's Balanced Salt Solution	
Opti-MEM I (1X)	
Fetal Bovine Serum (FBS)	GE Healthcare Life Sciences
Nitrocellulose Blotting Membrane	
DMEM	Sigma-Life Science
L-Glutamine	
Phosphate Buffered Saline tablet	
Dimethyl Sulfoxide (DMSO)	
Trypan Blue solution	
HEPES	
Hyaluronidase from sheep testes	
Dispase II	
Monoclonal Anti-Fibroblast Surface Protein Antibody	
BSA	
RIPA Buffer	
Protease Inhibitor Cocktail 3	
Phosphatase Inhibitor	
Collagenase A	
Trypsin	
Complement sera from rabbit	
DL-Dithiothreitol	
Bovine serum albumin	
Bicinchoninic Acid Solution (BCA)	
Sodium Dodecyl Sulfate (SDS)	
Ponceau S	
TWEEN® 20	
Trizma® Base	
Triton® X-100	
Bromophenol Blue	
Aprotinin	
Leupeptin	
EMEM	ATCC
Venor ® GeM One Step Test Kit (Mycoplasma)	Minerva Biolabs
XTT Cell Proliferation Kit II	Roche Molecular Biochemicals
PLD2 Expression Plasmid	OriGene
TransIT®-2020 transfection reagent	Mirus Bio
PLD2 protein siRNA	Ambion – Thermo Fisher Scientific

Negative control siRNA	
DharmaFECT 1 transfection reagent	Dharmacon
Primocin ™	InvivoGen
Copper (II) Sulfate Pentahydrate	AppliChem - PanReac
Glycine	
Methanol	
30% Acrymalide/Bis-acrylamide 29:1	Bio-Rad
Temed	
Precision Plus Protein ™ Dual Color Standards	
Ammonium Persulfate	
30% Glycerol Solution	
Non-fat powder milk	Nestle
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Scientific
SuperSignal™ West Pico Chemiluminescent Substrate	
Fluoromount G (for immunofluorescence)	Electron Microscopy Sciences
Paraformaldehyde 16% Solution, EM Grade	
[^{3}H] Butanol	American Radiolabeled Chemicals Inc.
Phosphatidylinositol 4,5-bisphosphate (PIP2)	Avanti Polar Lipids
Short side-chain phosphatidylcholine (PC8)	
Methanol for UHPLC	Fisher Scientific
Chloroform for UHPLC	
Toluene for UHPLC	
Methylene Chloride for UHPLC	
Acetonitrile for UHPLC	
Formic acid for UHPLC	
Isopropanol for UHPLC	

1.2. Commercial cell lines

The commercial cell lines used can be classified into three main groups: normal skin melanocytes (HEMn-LP, HEMn-MP, HEMn-DP), primary melanomas (A375, MEL-HO, Sk-Mel-28, Sk-Mel-31, G-361, ME4405) and metastatic melanomas (Hs294t, RPMI 7951, A2058, Sk-Mel-3, COLO-800, HT-144, WM-266-4, Sk-Mel-2, VMM1). In the following table, there is a detailed information of each cell line.

Table 7. Detailed description of the commercial cell lines used in this study.

Cell line	Code	Type	Tissue	Age	Sex	Company
HEMn-LP	C-002-5C	Lightly pigmented melanocytes	Foreskin	Neonate	Male	Cascade Biologics, Inc.
HEMn-MP	C-102-5C	Moderately pigmented melanocytes	Foreskin	Neonate	Male	Cascade Biologics, Inc.
HEMn-DP	C-202-5C	Darkly pigmented melanocytes	Foreskin	Neonate	Male	Cascade Biologics, Inc.
A375	ATCC CRL-1619	Primary melanoma	Skin	54	Female	ATCC
MEL-Ho	ACC-62	Primary melanoma	Skin		Female	Innoprot S.L.
Sk-Mel-28	ATCC HTB-72	Primary melanoma	Skin	51	Male	ATCC
Sk-Mel-31	ATCC HTB-73	Primary melanoma	Skin	33	Female	ATCC
G-361	ATCC CRL-1424	Primary melanoma	Skin	31	Male	ATCC
ME4405	CVCL_C680	Primary melanoma	Skin	83	Female	
Hs294t	ATCC HTB-140	Metastatic melanoma	Lymph node	56	Female	ATCC
RPMI 7951	ACC-76	Metastatic melanoma	Lymph node	18	Female	Innoprot S.L.
A2058	ATCC CRL-11147	Metastatic melanoma	Lymph node	43	Male	ATCC
Sk-Mel-3	ATCC HTB-69	Metastatic melanoma	Lymph node	42	Female	ATCC
COLO-800	ACC-193	Metastatic melanoma	Subcutaneous	14	Male	Innoprot S.L.
HT-144	ATCC HTB-63	Metastatic melanoma	Subcutaneous	29	Male	ATCC
WM-266-4	CRL-1676	Metastatic melanoma	Skin	55	Female	ATCC
Sk-Mel-2	ATCC HTB-68	Metastatic melanoma	Skin	60	Male	ATCC
VMM1	ATCC CRL-3225	Metastatic melanoma	Brain			ATCC

2. Methods

2.1. Cell culture

Cell culture is a widely used research tool to study the physiology and biochemistry of cells, as well as the toxicity and effects of different drugs and compounds in cells. This research model is established after the isolation of cells from a tissue and their maintenance in a beneficial artificial environment.

Cell cultures must be maintained in a controlled atmosphere of temperature, pH and humidity. In this way, they are kept inside an incubator, which creates a humid atmosphere with 5% CO_2 and a temperature of 37ºC, promoting cell growth. The manipulation of these cells must be done inside a laminar flow cabinet to provide a sterile environment and avoid contamination by bacteria, virus or yeast. Furthermore, all the solutions and materials used with the cells must be sterile and pre-heated at 37 ºC.

The culture conditions vary depending on the origin of each cultivated cells. According to their nutritional needs, each cell type is maintained in a medium of defined chemical composition that supplies growth factors, essential nutrients (amino acids, vitamins, carbohydrates, and minerals), hormones and gases (O_2, CO_2).

In this work, two different types of cultures were used. On the one hand, **primary cells** isolated directly from a nevus and established as a culture. Most of the primary cells have a limited lifetime, except primary cells isolated from tumors. On the other hand, we have also used commercially available **tumor cells**.

Table 8. Complete cell culture media composition for each cell line.

Cell line	Culture media
HEMn-LP	Medium 254 1X Human Melanocyte Growth Supplement
HEMn-MP	
HEMn-DP	
Nevus 1-9	
A375	DMEM 10% FBS 2 mM L-glutamine 100 UI/mL Penicillin 100 µg/mL Streptomycin
ME4405	
Hs294t	
RPMI 7951	
A2058	
HT-144	
Sk-Mel-28	EMEM 10% FBS 100 UI/mL Penicillin 100 µg/mL Streptomycin
Sk-Mel-31	
WM-266-4	
Sk-Mel-2	
MEL-Ho	RPMI 1640 GlutaMAX™ 10% FBS 100 UI/mL Penicillin 100 µg/mL Streptomycin
COLO-800	
VMM1	
G-361	Mc Coy´s 5A 10% FBS 2 mM L-glutamine 100 UI/mL Penicillin 100 µg/mL Streptomycin
Sk-Mel-3	

2.1.1. Defrosting cells

In order to initiate a cell culture from a purchased cell line or a stock created in the laboratory, first, the cells were revived. For that, the vial containing the cells was heated to 37 ºC and immediately mixed with 1/10 parts of complete cell media. The suspension was centrifuged at 200 g for 5 minutes at 4 ºC. Then, the supernatant was discarded and the cell pellet was re-suspended in 1 mL of complete cell media. Finally, the cell suspension was seeded in a 25-cm^2 culture flask with 4 additional mL of complete media.

2.1.2. Cell subculture

The cultured cells are growing actively, so after a few days they arrange in a monolayer and consume the nutrients present in the culture media releasing waste products of cell metabolism. Therefore, when 90% confluence was reached, the cells were harvested and plated at a lower density with fresh complete media, to maintain the cell culture over time.

To subculture the cells, cell culture media was removed and the cells were washed with phosphate saline buffer (PBS) without calcium and magnesium to favor the activity of the dissociating agent. Then, the cells were detached by the action of the trypsin-EDTA solution for approximately 5 minutes at 37 ºC. Once the cells were detached, fetal bovine serum (FBS) was added to inactivate the dissociation agent trypsin and the solution was centrifuged at 200 g for 5 minutes. Subsequently, the supernatant was discarded and the cells re-suspended in 1-2 mL of fresh culture media. For cell counting, the cells were mixed 1:1 with trypan blue, which is a vital stain that selectively colors the dead cells in blue while the living cells remain uncolored. Then, using the TC20 Automated Cell Counter (BioRad), the total number of cells and their

viability were determined. In this way, the volume of cell suspension necessary in each experiment was calculated, together with the viability of the cells, which must be higher than 90% to carry out any analysis.

2.1.3. Freezing cells

The cells cannot be maintained eternally in culture, since they are prone to suffer genetic alterations, microbial contamination, etc. Therefore, it is extremely important that the cells are frozen at low passage and a seed stock is made for long-term preservation in liquid nitrogen. Thus, if the cells were maintained in culture for about 20 passages or were contaminated, they were replenished from frozen stock.

Following the same procedure as for subculturing cells, after centrifugation, the cell pellet was re-suspended in freezing media (10% DMSO, 90% FBS) at the recommended cell density. Then, the cells were frozen slowly by lowering the temperature by approximately 1 ºC per minute using a "Mr. Frosty" cryo-freezing container in a -80 ºC freezer. After 24 h, the frozen vials were transferred to liquid nitrogen for long-term storage.

2.1.4. Mycoplasma detection

While bacterial and yeast contaminations can be easily detected in cell cultures, mycoplasma contamination is difficult to recognize, since they are not detectable by the eye or by light microscopy. Moreover, antibiotics used regularly in cell culture are not effective for these microorganisms. It is expected that between 10% and 85% of the cell lines used in any laboratory are contaminated (InvivoGen). This contamination can have many effects on cells, such as chromosomal aberrations, alterations in the proliferation rate, cell metabolism and cell viability. Hence, cell cultures must be tested every 3 months.

For this, Venor® GeM One Step Test was used, which through PCR technique detects 10 different species of mycoplasma in culture media that have been in culture for at least 48 h. If it was positive for mycoplasma, the cells were discarded and new cells were revived from the stock in liquid nitrogen.

2.1.5. Cell pellet collection

Once sufficient amount of cells was reached, they were harvested and collected to store the cell pellet and use it later for the extraction of proteins, lipids and cell membranes.

When 90% confluence was achieved, as in the cell subculture, the cells were harvested with trypsin and centrifuged at 200 g for 5 minutes. Then, the supernatant was removed, the pellet was re-suspended in 1 mL of PBS and the cells counted. This suspension was centrifuged again at 300 g for 5 minutes. After discarding the supernatant, the cell pellet was introduced in liquid N_2 for rapid freezing, and immediately after, they were stored at -80 ºC until use.

2.1.6. Cell transfection

Cell transfection is the procedure by which foreign genetic material is introduced into eukaryotic cells for two different purposes. On the one hand, to induce gene expression to increase the cellular levels of a recombinant protein a plasmid or mRNA is used. On the other hand, knockdown of a certain gene to reduce the expression of a protein RNA interference is used. Furthermore, the transfection can be stable since the foreign material integrates the genome or transient for a limited time of 24 to 96 hours. This process can be achieved through different chemical, biological or physical methods. In this study, lipid-based reagents forming nucleic acid-lipid complexes were used via electrostatic interactions between the negatively charged nucleic acids and the cationic lipids. These complexes were captured by the cells as they fused together

with the phospholipids of the cell membrane and delivered the genetic material inside the cell. Once inside the cell, the transfected DNA was translocated into the nucleus while the RNA remained in the cytoplasm.

- PLD2 protein overexpression:

A commercially available expression plasmid was employed to increase the synthesis of PLD2 protein in melanoma cells. The plasmid increased the amount of messenger RNA of PLD2 gene and this resulted in higher levels of PLD2 protein in the cells.

Melanoma cells were seeded in a 6-well plate, and when they reached 60-70% confluence, they were transfected with the expression vector. At this point, new complete media was added to the cells along with the transfection mixture. The latter was composed of 1 µg of DNA and 2 µL of TransIT®-2020 transfection reagent mixed in 300 µL of Opti-MEM™ for 20 minutes prior to adding it to the cells. In another well, the cells were only incubated with the transfection reagent plus a scramble plasmid, which was used as a control condition of the transfection.

After 48 hours of incubation, the transfection media was removed and the cells were ready for any experiment. The efficacy of the overexpression was verified by western blot.

- PLD2 protein silencing:

In order to knockdown the expression of the PLD2 protein, a commercial siRNA was used. This methodology is based on a double-stranded RNA molecule of 20-25 base-pair in length that interferes with the messenger RNA of a certain gene that blocks its translation into protein and degrades it.

When the cultured cells reached 60-70% confluence in 6-well plates, the cell medium was removed and fresh complete medium was added. Immediately, the transfection mix was formulated as follows: 150 µL Opti-MEM™ and 50 nM of siRNA were mixed in a glass tube; in another tube, 150 µL Opti-MEM™ and 9 µL RNAiMax transfection reagent were mixed. After 5 minutes of incubation, both tubes were mixed and incubated for another 5 minutes before adding the solution to each well. After 48 hours of incubation, the cells were ready for any experiment. For the transfection control condition, an irrelevant siRNA molecule was used, which has been shown to have no effect on proliferation, viability or cell morphology. The silencing efficacy was verified by western blot.

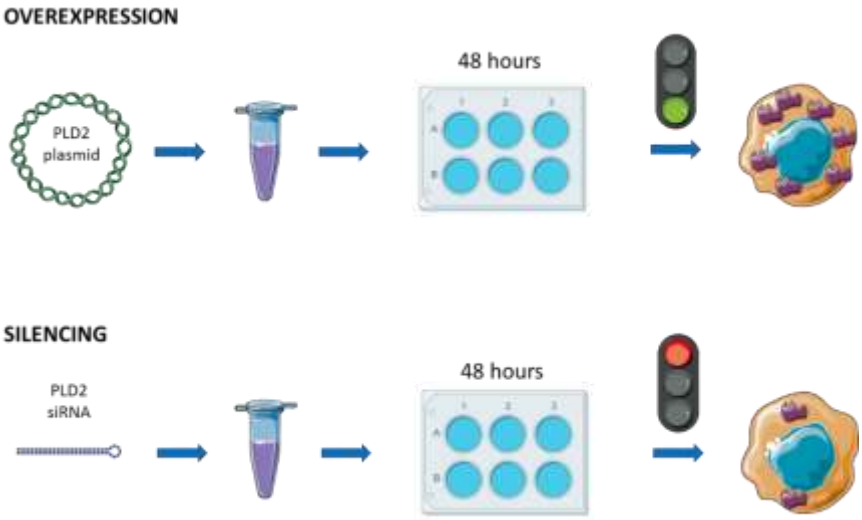


Figure 22. Workflow schema of PLD2 overexpression and silencing.

2.1.7. Cell proliferation assay

In order to study cell proliferation, the XTT Cell Proliferation Kit II was used. This is a colorimetric assay whereby only viable cells are metabolically active to reduce the tetrazolium salt XTT of yellow color in an orange colored formazan dye, which can be detected at a wavelength of 490 nm by a spectrophotometer. The amount of formazan generated is directly proportional to the number of viable cells.

First, the cells were seeded in triplicates in flat-bottom 96 well-plates and left overnight to allow their adhesion to the surface. Then, they were starved for 2 hours using a culture media supplemented with 1% FBS to synchronize the cell cycle of all the cells present in the culture. Afterwards, the starving medium was removed and complete medium was added to the cells. 24 hours later, the XTT mixture was added to the cells at a ratio of 1:50. After 4 hours of incubation, the absorbance of each well was measured at a wavelength of 490 nm in a spectrophotometer. The proliferation rate of the cells studied was calculated as a percentage in relation to the control.

2.1.8. Cell invasion assay

Metastatic cells have the ability to detach from the primary tumor and by degrading the extracellular matrix invade the surrounding stroma. To evaluate the invasion capacity of the cells, an *in vitro* transwell invasion assay was performed. The upper chamber of the transwell is sealed with a polycarbonate filter with pores of 8 µm diameter and coated with Matrigel® that resembles the extracellular matrix in tissues, since it contains collagen IV, laminin, entactin, heparan sulfate and proteoglycans. Invasive cells degrade the Matrigel® and pass through the pores to reach the underside of the filter.

First, starving media without FBS was added to the transfected cells for 2 hours. At the same time, Matrigel® plates were taken out of the fridge and allowed to reach room temperature. Then, the Matrigel® from the transwell inserts was rehydrated by adding starving media to the upper and lower chambers for 2 hours.

After this period, the starving medium of the inserts and the wells was carefully aspirated, without touching the Matrigel® layer. At that time, complete cell culture medium was added to

the bottom well. Simultaneously, the cells were harvested and added to the upper chamber at a concentration of $7.5x10^5$ cell/mL in starving media with 0.5% FBS. This was incubated for 20h in the culture incubator.

Eventually, the non-invasive cells that remained in the transwell insert were removed by aspirating the cell medium and scrubbing the filter with cotton swabs. Then, the media in the well was replaced with 4% paraformaldehyde (PFA) to fix the cells that invaded and reached the other side of the filter, as after 20 hours of invasion assay most of the cells are still on the filter and have not yet reached and attached to the bottom of the well. After 10 minutes and three washes of PBS, the invading cells were stained with 0.2% crystal violet for 8 minutes by adding the solution to the bottom well. Then, the transwell was washed with water as many times as necessary to remove all the non-specific staining. When the inserts were dry, they were observed in the microscope and 6 different fields were photographed at 20X. Finally, all the cells in each field were counted and the percentage of invasiveness of each group was calculated related to the control.

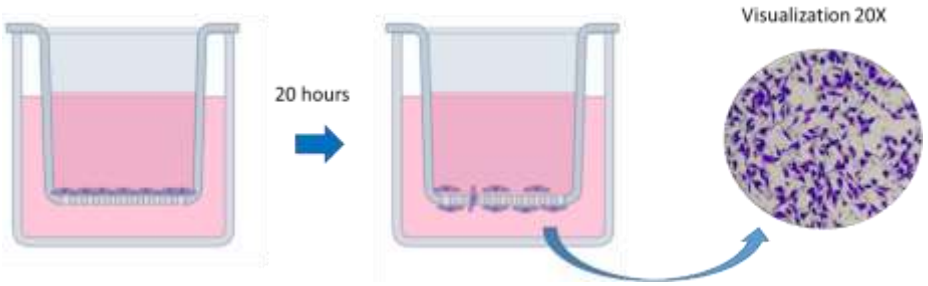


Figure 23. Scheme of the transwell *in vitro* invasion assay, and eventual visualization of the cells that invaded.

2.1.9. Cell migration assay

Similarly to invasion assay, the migratory capacity of melanoma cells was studied using boyden chambers. However, in this case, the transwell with 8 µm pores was not coated with matrix.

First, the transfected cells were starved for 2h. After this period, the cells were harvested and added to the upper chamber at a concentration of $5x10^4$ cell/mL in 0.5% FBS media. At the same time, complete culture media was added to the bottom chamber.

After 20 hours of incubation, the non-migrated cells were carefully aspirated and the upper filter scrubbed with a cotton swabs. Then, the culture media in the bottom chamber was substituted with 4% PFA to fix the migrated cells in the bottom side of the filter. After 10 minutes and washing the cells 3 times with PBS, the cells were stained with 0.2% crystal violet for 8 minutes. Then, the transwell was washed with water until all non-specific staining was removed. Eventually, the filter was cut out with a scalpel and mounted on a microscope slide. As such, the migrated cells could me observed in a microscope at 20X and six different fields were photographed. Then, all the cells in each field were counted and the percentage of migration for each group was calculated according to the control.

2.2. Melanocyte isolation from human nevus

For this, human nevus from different patients were used, and the pertinent ethic statement was applied for the collection of these samples. The Euskadi Ethics Committee (OncoImage, 14-10) approved this study and written informed consents were obtained from all the subjects.

As soon as the nevus was surgically removed in the operating room, a piece of it was placed in 10 mL of sterile cell culture medium with antibiotics. Henceforth, the handling was performed under aseptic conditions and all the solutions and materials used were sterile.

In order to eliminate surface contamination of the patient's skin, the tumor was introduced into ethanol 70º for 15 seconds several times and washed in PBS. Then, it was incubated overnight at 4 ºC in an enzymatic solution of 0.25% trypsin, 20 mM HEPES and Primocin™ antibiotic solution in Hank's Balanced Salt Solution (HBSS).

Subsequently, the dermis and epidermis were separated in a petri dish using a scalpel; the epidermis was discarded, while the dermis was diced into millimeter pieces. These small portions were then incubated in an enzymatic solution of 0.05% Collagenase, 1.25 U/mL Dispase II and 0.1% Hyaluronidase for 2h while stirring in a water bath at 37 ºC.

Once the fragments were partially digested, a mechanical digestion followed the isolation process. For this, the pieces were passed through a 40 µm pore filter that was adapted to a 50 mL tube, and the filtrate was centrifuged at 350 g for 5 minutes. Then, after discarding the supernatant, the cell pellet was re-suspended in Medium 254 and seeded in a petri dish. After an overnight incubation, the culture was washed several times with PBS until all the non-adhered cells (death cells, lymphocytes, erythrocytes, etc.) were removed and fresh medium was added.

In this culture, there were mostly melanocytes and fibroblasts. Willing to have a pure culture of melanocytes, when the cells reached confluence they were washed with 20 nM HEPES in HBSS solution and treated with an antibody (1:500) that recognized a protein present in the fibroblast membrane for 1 hour at 4 ºC and gentle agitation. Without delay, the culture was cleaned three times with 20 nM HEPES in HBSS solution at 4 ºC. Afterwards, anti-rabbit complement (1:2) diluted in a cold 20 nM HEPES in HBSS solution was added and incubated for 4 hours at 37 ºC with vigorous shaking. In this way, the complement bound to the anti-fibroblast antibody caused the lysis of the fibroblasts. Eventually, the culture was rinsed several times to remove the remaining complement and Medium 254 was added to continue with the culture of pure melanocytes.

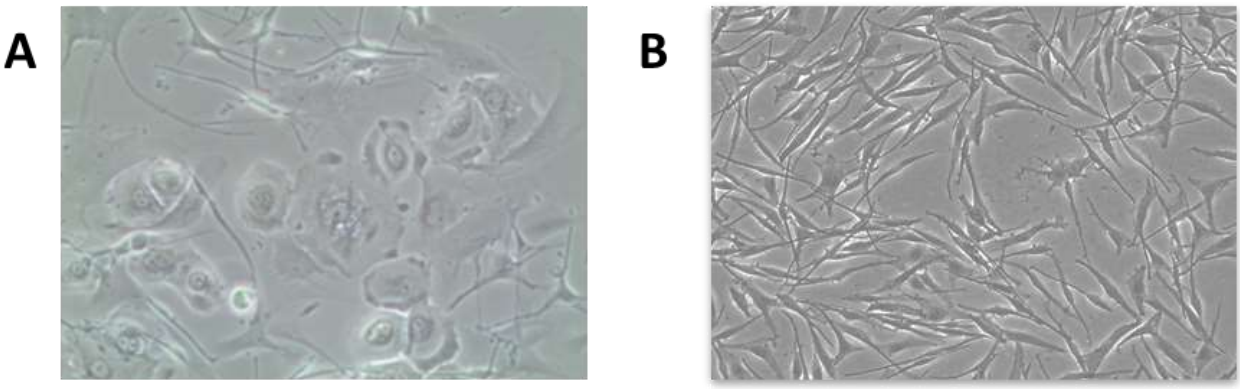


Figure 24. Nevus melanocyte isolation process at different steps. **A.** Co-culture of melanocytes, fibroblasts and keratinocytes. **B.** Culture of pure melanocytes., fibroblasts and keratinocytes. **B.** Culture of pure melanocytes.

2.3. Cellular lipids analysis

2.3.1. Sample homogenization

The stored cell pellets were homogenized in 1 mL of PBS using the Polytron homogenizer applying 4 cycles of 10 seconds of homogenization and 10 resting seconds. During the process, the samples were kept on ice. At this point, 10 µL of each sample was extracted for protein quantification by the BCA assay and the rest was saved for lipid extraction.

2.3.2. Protein quantification

The protein concentration of each homogenate was determined using the BCA colorimetric assay (Bicinchoninic acid assay). This method is based on the Biuret test. The peptide bonds of the proteins in an alkaline solution reduce Cu^{+2} from copper (II) sulfate to Cu^{+}. The amount of Cu^{+} generated is proportional to the protein content. Two BCA molecules chelate with one Cu^{+} ion and form a purple complex that can be detected at the wavelength of 562 nm with a spectrophotometer[148].

For this, a standard BSA curve (0-10 mg/mL) was used. Once all the study samples and the standard curve samples were ready, the working reagent was prepared, where 50 parts of bicinchoninic acid were mixed with one part of $CuSO_4$ solution at 4% (copper sulfate pentahydrate). In a 96-well plate, in triplicate, 10 µL of each condition was added to each well along with 200 µL of the working reagent. This was incubated for 30 minutes at 37 ºC in the dark.

Eventually, the absorbance of each well was read on a spectrophotometer at the wavelength of 562 nm. Based on the absorbance obtained for the standard curve, the protein concentration of each sample was calculated.

2.3.3. Lipid extraction

Cell lipid extraction was carried out in the laboratory of Professor Begoña Ochoa. This assay was based on the Bligh & Dyer protocol[101], using the same amount of protein for each sample. All the glass material used for this methodology must have been previously treated with a chromic mixture (10 g of potassium dichromate diluted in 1 L of dH_2O and 1 L of sulfuric acid) and thoroughly rinsed, in order to clean the glass material and remove all the residues.

Inside a ground neck flask, 0.4 mL of homogenate, 2 mL of chloroform and 4 mL of methanol were vigorously vortexed for two minutes. Immediately, 2 mL of H_2O and 4 mL of chloroform

were added, and the solution was stirred again for another two minutes. The mixture was then centrifuged at 200 g at 4 ºC for 15 minutes, achieving phase separation. The lower phase was transferred to another glass tube and saved for later. The upper protein phase was re-extracted by adding 1.6 mL of chloroform, methanol and H_2O. This solution was vigorously vortexed for 2 minutes and centrifuged at 200 g at 4 ºC for 10 minutes. At this point, the upper aqueous phase was discarded and the lower phase mixed with the previous chloroform phase. Then, in order to evaporate the chloroform and concentrate the extracted lipids, the mixture was introduced in an evaporator/concentrator, and dry lipid-extracts were obtained. To prevent oxidation of the lipids, the tubes were closed under a stream of N_2. As the aqueous phase was tossed away, the more polar lipids were not analyzed using this extraction method.

Next, the solvent was evaporated in an evaporator/concentrator system for about one hour, until the extract was dried. This extract was re-suspended in 900 µL of chloroform/methanol (2:1). The solution was transferred to a UHPLC vial and the solvent was again evaporated. Each tube with the lipid extract was closed under N_2 atmosphere and stored at -80 ºC until use.

2.3.4. Lipidomic analysis

The Central Analysis Service of the UPV/EHU (SGIKER) carried out this analysis. For the identification and quantification of lipids, an untargeted mass spectrometry technique was performed: UHPLC-ESI-Q-TOF (Ultra-high performance liquid chromatography coupled with time of flight quadrupole tandem mass spectrometer).

- Ultra-High Performance Liquid Chromatography (UHPLC):

For a better identification of the lipids, a reverse-phase UHPLC technique was used to separate the lipid families, with a non-polar stationary phase composed of silica beads and two different polar mobile phases (A and B), which were mixed at different percentages during the elution process.

First, the dry lipid extracts were reconstituted in 150 µL chloroform/methanol 2:1. Then, 7.5 µL of this solution was injected into the mobile phase and introduced into the chromatograph.

In order to ensure the accuracy of the results obtained in this experimental strategy different quality control samples and internal standards were analyzed together with the study samples. First, three blank samples were injected, consisting of $CHCl_3$ (2:1 v/v). Then, the 34 study samples were randomly introduced in the system, together with an internal standard solution of 1.92 µM PC (17:0/14:1) and 1.68 µM PI (17:0/14:1) diluted in $CHCl_3$ (2:1 v/v), which are detected in positive and negative ionization mode, respectively. In addition, 15 system Quality Control (QCsys) were introduced for the reconditioning of the system before the analysis of the study samples. QCs were prepared by reconstituting in 150 µL of $CHCl_3$ (2:1 v/v) the lipid extracts of four additional melanoma cell lines that were not analyzed in this experiment. Besides, QC samples (QCsample) were also analyzed. These were prepared by adding 15 µL of all the study samples, thus containing all the lipids that would be identified in the study samples. They were used to determine all the lipids that would be detected in the study samples, and also, as they were injected at the beginning, during the study sequence, and at the end of the analysis, to determine the analytical variability of the identified lipids, and normalize the results (see the injection order of all the samples in **Figure 25**.

At the beginning of the chromatography approach, the mobile phase was composed of phase A (acetonitrile and water 40:60) in 60% and phase B (acetonitrile and isopropanol 10:90) in 40%. Therefore, the first analytes eluted were the polar ones. Progressively, the concentration of

phase B was increased, until reaching 100%, when the non-polar compounds were eluted and directly entered the mass spectrometer.

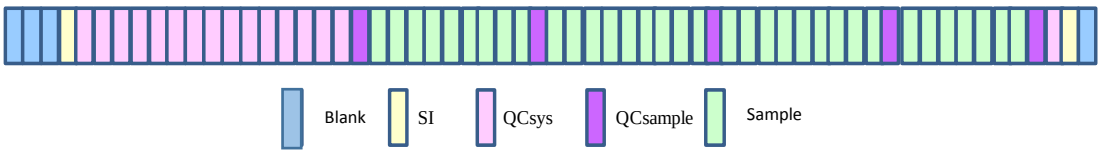


Figure 25. Injection order of the samples analyzed.

- ESI-TOF Mass spectrometry:

The lipid molecules were ionized in the ionization source using ESI methodology (ElectroSpray Ionization). The ions were then targeted through a tandem Q-TOF mass analyzer (Quadrupole/Time-of-flight) that separates the molecules according to their mass-to-charge ratio.

Eventually, the ions enter the detector, which records the information of both the precursor and the fragmented ions and their abundance in the mixture, which is then sent to the data system where it is graphically represented as mass spectra. Each peak of the graph represents a specific mass present in the mixture, and the amplitude of each peak represents the relative abundancy of that ion.

Table 9. UHPLC-ESI-Q-TOF analysis conditions.

UHPLC	MS
Column: Acquity UHPLC HSS T3 2.1x 100 mm, 1.8 µm (Waters)	**Ionization mode:** ESI positive/negative
Column Temperature: 65 ºC	**Adquisition mode:** MS^E in resolution mode (FWHM≈20.000)
Flux: 500 µL/min	**Capillary voltage:** 0.7kV (ESI+) & 0.5 kV (ESI-)
Mobil phase A: Acetonitrile/H_2O (40:60) with 10 mM NH_4Ac	**Cone voltage:** 35 V
Mobil phase B: Acetonitrile/Isopropanol (10:90) with 10 mM NH_4Ac	**Source temperature:** 120 ºC
Gradient: 0-10 min, from 40 to 100%B; 10-11min, 100%B;	**Desolvation temperature:** 400 ºC
Temp. automatic injector: 4 ºC	**Desolvation gas:** 900 L/h
Injection volume: 7.5 µL	**Gas of the cone:** 30 L/h
	Adquisition range: 50 a 1200 u.
	Scan time: 0.5s

- Data analysis:

First, for the lipid assignment, in order to generate a data matrix, the R-software 3.1.1. was used. Then, for the identification of the lipids, the MSe Data viewer was employed to process the data obtained by the R software, and, finally, using the SimLipid software the lipid assignment was made.

Then, before performing any statistical analysis, the data obtained for each variable were normalized and logarithmically transformed to achieve a Gaussian distribution, and therefore avoid systemic bias in the multivariate analyses performed later.

To detect the differences between the samples studied, different multivariate data analysis were performed using the SIMCA 14.1 software. First, a **principal component analysis** (PCA) was conducted.This is a non-supervised analysis that finds patterns within the data without reference whether they belong to the same study group. It aims to find groups of similar samples, detect abnormal samples, relevant variables and the relation between variables and samples. For this, it transforms the data into fewer dimensions (Principal Components) that are representative of the features of the data, and better summarizes it using the fewer number of principal components possible. This is, only the more informative dimensions are used. To visualize the distribution of the data, score scatter plots of two principal components can be performed, where positively correlated samples are close to each other and the non-correlated data are far from each other.

In order to validate the PCA model, there are some model diagnostic tools. Like in a regression, the model fit is represented by R2, and it informs how well the data can be mathematically reproduced by the computed model. Hence, if the correlation is good R2 is close to 1, and gets worse when R2 is smaller. However, it is not sufficient to have a R2 close to one to validate the model, as also its predictive ability is important. The latter is estimated by Q2, which represents the lack of model fit of each sample to the PCA model. It measures the variation after applying the created model to the samples, this is, how much of a sample cannot be explained by the model. When Q2>0.9 the fitting to the model is considered excellent.

Besides, Hotelling's T2 analysis is also employed to evaluate the PCA model obtained. It measures the variation within the PCA model, by indicating how far each sample is from the center of the model. It sets a confidence limit were the samples can lie. In this study, if any result lies outside the limits of 95% of confidence interval after applying Hotelling's T2, it will be considered a strong outlier. To visualize it, a score scatter plot of the two principal components was performed.

On the other hand, the detection tool of moderate outliers is DModX, which stands for distance to the model in X-space, with a maximum tolerable distance (DCrit) that is usually set in 0.05. Moderate outliers have DModX values larger than DCrits, so do not fit the model well.

The next step was to perform different supervised analyzes. First, a **partial least squares discriminant analysis** (PLS-DA) was carried out. This study analyzes the differences between each study group according to their lipidic profile, and is commonly used in metabolomic studies for classification and biomarker identification, as it sharpens the differences between the study groups. This model is especially useful when PCA method is not able to stablish differences between the study groups. As the groups within the samples are already known, the aim of this analysis is to determine if the study groups are actually different, and what are the variables that stress the differences between the groups.

For the validation of the model, R2, Q2 and Hotelling's T2 ellipse plot are used as well as in PCA. Besides, permutation testing is also used in this case, which allows to determine the ability of the model to predict the Y response of new samples examined. Here, the variable Y was permutated 999 times in the two studied classes in each analysis.

Moreover, in order to validate the significance of the differences observed in the PLS-DA models, the p-values of each comparison were calculated using ANOVA approach.

The comparisons of the intensities detected for each lipid subclass were performed by adding the intensities obtained for all the lipid species pertaining to that lipid subclass. Then, the sums obtained for each study group were compared relating them to the value obtained for skin melanocytes. This is, the sum of the intensities of all the PCs in one group was divided by the sum of the intensities of all the PCs in skin melanocytes.

The next analysis was **orthogonal partial least squares discriminant analysis** (OPLS-DA). This model forces the variation between the groups, in order to detect the molecules that define the differentiation between the experimental groups. In fact, this method is used to identify the species that generate relevant changes in the lipidome. For this, a regression model is constructed between the multivariate data (X) and the class information (Y). To visualize the discriminating variables and thus select the potential biomarkers, score plots were used. In addition, jack-knife confidence intervals of the loading plots to reduce bias of parameter estimated and to determine variance were utilized, and also VIP (Variable Importance in Projection) plots. The latter graphs visualize the relative contribution of each lipid molecular species to the variance between each study group. High values of VIP score show great contribution of that lipid to the group separation.

The significantly different lipids detected were represented in Box-whisker plots, in order to study their relative abundance in each study group.

- Lipidomic analysis limitations:

The analytical procedure followed in this experiment only gives a hint of the tendency of the changes within the cells. First, in the extraction step, the more polar and hydrophilic lipid species were missed as they were dissolved in the aqueous phase, which was discarded. Concretely, all the phosphorylated lipids such as phosphorylated PIs, and complex glycolipids, specially gangliosides, were dissolved in the aqueous phase and therefore missed. These lipid species are thought to be very important in some malignant transformations, so, for a global lipidome analysis, in future experiments, the most polar lipids should be extracted using different methods.

In addition, this is a semi-quantitative analytical procedure, as internal standards for quantification have not been used. The comparisons carried out with these results are only the comparative of the intensities of each lipid species as the mean of all the cell lines of each study groups. This is, one cannot compare the abundancy between different lipid classes, as the intensities vary according to the adducts formed for the mass spectrometry approach, which are very different depending on the lipid species. Hence, for example, we cannot know if there are more phospholipids than sphingolipids in a concrete cell type, but we can determine if there are more phospholipids in one group (melanocytes, e.g.) than in other (primary melanoma, e.g.).

2.4. Cell membrane analysis

Employing functional cell membrane microarrays, cell membrane lipids were studied. This is a new biotechnological tool developed by IMG Pharma S.L., which, using specific printing protocols, maintains the lipid environment of the membranes, so that the lipid profile of the samples can be determined. In addition, hundreds of samples can be immobilized on the same pre-treated microscope glass slide, allowing the analysis of all of them at the same time, saving time and expenses. Specifically for the analysis of lipids, a mass spectrometry technique was applied on the membranes immobilized in the microarrays.

2.4.1. Cell membrane isolation

Using a Teflon-glass grinder and Ultra-Turrax® disperser, cell membranes of the stored cell pellets were isolated using a 50 mM Tris buffer supplemented with 1 mM EGTA, 3 mM MgCl2 and 250 mM sucrose. After centrifuging the suspension for 5 minutes at 40 g, the resulting supernatants were centrifuged again at 18,000 g for 15 minutes at 4 ºC. Then, the supernatants were discarded and the pellets washed in 20 volumes of 50 mM Tris buffer and centrifuged again at 18,000 g for 15 minutes at 4 ºC. Once again, the supernatant was discarded and both pellets mixed. Finally, the protein concentration was determined using the Bradford method. The cell membrane preparations were stored at -80 ºC until use.

2.4.2. Protein quantification

Protein quantification of the cell membrane preparations was performed by the Bradford protein quantification assay[149]. This is a colorimetric analytical procedure that is based on the color shift of the Coomassie Brilliant Blue dye in an acidic environment, which changes its color from red to blue, after binding to the protein present in the solution. In the absence of protein, the dye remains red and its absorption spectrum is at 465 nm. However, the blue dye bound to the protein has the maximum absorbance at 595 nm, so the experiment was carried out at this wavelength.

First, a BSA standard curve (0-0.1 mg/mL) was prepared. Then, in a 96 well-plate in triplicate, 10 µL of each sample and standard curve condition were mixed with 250 µL of Bradford working solution (0.01% Coomassie Brilliant Blue, 0.05% Ethanol 95º, 0.1% Phosphoric Acid). After 10 minutes of agitation in the dark, the absorbance was analyzed at 595 nm. Using the results obtained in the Bradford method, all the samples were diluted to the same protein concentration, in order to immobilize the same protein quantity in each spot of the array.

Then, after the printing of the samples, the protein concentration of each spot of the array was measured. For this, the slides with the cell membrane preparations printed on them were introduced in the Bradford working solution for 10 minutes and washed in water for 5 minutes. Then, the color intensity for each spot was determined. This is used as a quality control of the printing, as all the spots representing different samples must have similar protein concentration.

2.4.3. Microarray development

Three normal skin melanocyte cell lines, nine melanocyte cell lines isolated from nevus, six primary melanomas and nine metastatic melanoma cell lines were placed. Here, replicates of three different passages of each sample were printed.

All cell pellets were diluted to the same concentration and printed on expressly pre-treated glass slides by a non-contact microarrayer (Nano_plotter NP 2.1). The piezoelectric tip dispenses 4 nL per dot, and in this case, 20 drops per sample were spotted. The microarrays were stored at -20°C until use.

After printing the arrays, three different slides from each batch were randomly chosen and stained using the Bradford method. This enables to calculate the protein concentration in each spot. Nonetheless, in the same microarrays together with the samples, different concentrations of BSA were printed, in order to establish an internal calibration curve that allows the transformation of the intensities of each spot for the Bradford into nanograms of proteins. In this way, the results obtained in the lipidomic assay can be normalized to the protein quantity on each spot.

2.4.4. Lipidomic analysis

The research group of Professor J.A. Fernandez of the UPV/EHU carried out this analysis. It is based on Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry using a LTQ-Orbitrap XL analyzer.

- MALDI mass spectrometry:

For the preparation of the sample, first, the microarrays were allowed to reach room temperature. Then, using a glass sublimator (Ace Glass 8023) the matrices were placed on the samples, using MBT (2-Mercaptobenzothiazole) for positive ionization or DAN (1,5-Diaminonaphthalene) for negative ionization. In this way, uniform thin layers are formed, allowing the scanning of the molecules in positive or negative ion mode for several hours.

Next, using the MALDI methodology and a N2 laser as ionization source (100 μJ maximum power, elliptical spot, 60 Hz repetition rate), the molecules were ionized, irradiating the spots with the laser, inducing absorption by the matrix in the wavelength of the laser and desorption of the sample and the matrix with minimal fragmentation. Then, using a LTQ-Orbitrap XL analyzer, trapped ions were detected and a mass spectrum was generated. This was provided as an image, where each peak was mapped along the sample. Data was acquired both in positive and negative ionization-mode with 150 μm spatial resolution and a mass resolution of 60.000 FWHM at m/z=400, and with a laser energy between 20-30 μJ/pulse. All the data acquired was in the range between 550-1200 units in the negative ionization mode and 480-1000 units in the positive ionization mode.

In order to ensure the reproducibility of this methodology, and thus, of the data obtained, five different arrays were analyzed in different days for both ionization modes. Besides, the spots corresponding to the melanocytes were printed in four different locations of the array to check if the results were the same in all the locations of the slides.

- Lipid assignment:

Before the identification of each mass channel detected, all the spectra were aligned and normalized to the total iont current (TIC). Then, each ion channel was assigned to a concrete lipid molecule using an in-house synthetic database, constructed by the combination of side-chains and polar head groups of lipids, with more than 30,000 entries. Next, the MALDI assignments were compared to the ones achieved in UHPLC-MS/MS, and the peaks that correspond to the matrix, lipid fragments (PA and CerP), matrix adducts and isotopic distributions were removed. Eventually, PCs, ether PCs, PEs, ether PEs, PSs, PGs, ether PGs, PIs, ether PIs and SMs were detected in MS^-, while PCs, ether PCs, ether PCs, PEs, ether PEs, HexCer, SMs, DGs and TGs are identified in MS^+.

In addition, as UHPLC-MS/MS gives better description of the side-chains structure of the lipids, if the molecule was detected in both methodologies the nomenclature of UHPLC-MS/MS was used thereafter.

- Statistical analysis:

For the statistical analysis, SPSS Software was used. The first approach was to conduct a t-test comparing the intensity detected for the different lipid molecular species between normal melanocytes (skin + nevus) and melanoma cells (primary + metastatic). Next, a multi-comparison paired t-test between all the groups was carried out ($p<0.05$), which compared each lipid specie in a paired analysis as follows: skin vs nevus melanocytes; skin melanocytes vs primary melanoma; nevus melanocytes vs primary melanoma; skin melanocytes vs metastatic melanoma; nevus melanocytes vs metastatic melanoma; primary vs metastatic melanoma. For the post-hoc correction, Levene's test was conducted to assess the equality of the variances and choose the correct post-hoc method, either Bonferroni or Games-Howell. If the significance of Levene's test was $p<0.05$, the null hypothesis of equal variances was rejected, and it was concluded that there is a difference between the variances in the population. In this case, Games-Howell must be applied. However, if $p>0.05$, the null hypothesis is assumed and Bonferroni is conducted.

2.5. Protein analysis

2.5.1. Western blot

This widely used analytical tool allows the separation by the molecular mass of proteins within a complex mixture using gel electrophoresis and the subsequent detection of specific proteins by immunoblotting with specific antibodies. With this procedure, the molecular weight of the protein of interest can be estimated, study if it has post-translational modifications such as phosphorylation and ubiquitination, and a semi-quantitative estimate of the protein levels can be made by comparing all the samples studied.

- Protein extraction:

Protein extraction was performed from stored cell pellets or from newly prepared cultured cell pellets. If stored cell pellets were used, they were first kept on ice for 10 minutes until they were thawed. For protein extraction, 250 µL of RIPA Buffer was added per 5 x 10^6 cells. This buffer is composed of 150 mM sodium chloride, 1% IGEPAL®ca-630, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris-HCl at a pH of 8. Additionally, 1X protease inhibitor cocktail-3 and 1X phosphatase inhibitor were added to protect the sample, since as soon as the lysis begins, the proteolytic and dephosphorylating processes start.

After vigorously re-suspending the samples in the lysis buffer, the solution was kept on ice for 15 minutes. In this process, the cellular DNA escapes from the nucleus and a cloud of high-density DNA is formed. Considering that it would interfere with the experiments, this was broken by passing the solution through a 20G needle. Immediately, the solution was centrifuged at 15,000 g for 5 minutes at 4 ºC. The supernatant was transferred to a new tube, since the proteins of interest were dissolved there. Cell lysates were stored at -20 ºC until use.

- SDS-polyacrylamide gel electrophoresis (SDS-PAGE):

The SDS-PAGE technique separates proteins according to their molecular weight, based on the ability of charged molecules to move in an electric field. Prior to electrophoresis, the samples are denatured by heat in the presence of a denaturing agent such as SDS. This detergent binds to the proteins destroying their secondary and tertiary structure, and covering their intrinsic charge, conferring them a negative charge. One SDS molecule binds to two amino acids, so the mass-charge-ratio would be similar in each protein analyzed. Reductive electrophoresis was run, so that reducing agents such as DTT (dithiothreitol) were added. The denaturation was completed by DTT as it breaks the disulfide bonds of the proteins. In this way, when voltage was applied, all the proteins migrated from the cathode (-) to the anode (+) at different speed depending on their molecular weight.

For the separation, a gel of polyacrylamide was used, which was constructed by crosslinking acrylamide and bis-acrylamide molecules. This polymerization was initiated with ammonium persulfate and catalyzed with temed (N,N,N,N´-tetrametilnediamina). Depending on the concentration of acrylamide and bis-acrylamide used, different pore sizes were formed within the gels. Thus, the higher the concentration of acrylamide, the smaller the pore size and vice versa. In this way, if the protein of interest has a high molecular weight, a low concentration of acrylamide should be used for a higher resolution. However, the higher the acrylamide concentration, the more separated the lower molecular weight proteins will be.

Table 10. Percentage of acrylamide needed to resolve the proteins according to their molecular weight.

Acrylamide %	15	12	10	7.5
kDa range	12-45	10-70	15-100	25-200

The gels used for western blot are composed of two different parts. The biggest is the running. Here, the proteins are separated, so that depending on the molecular weight of the proteins of interest, the percentage of acrylamide used can be adapted. The top is the stacking, which is a small portion where all the proteins are concentrated and begin to migrate at the same time through the running gel. It is always prepared at the 4% of acrylamide.

For casting the gels, first, the shorter and taller glass plates were cleaned with 70% ethanol and water. Then, they were introduced into the casting frame, which was placed in the casting support. When the apparatus was assembled, the running gel solution was prepared and added inside both glass plates, leaving 2-3 cm from the top. After that, water was added in order to explode the bubbles that could have formed and achieve a straight surface.

Table 11. Composition of the running and stacking gels relying on the acrylamide percentage.

	RUNNING GEL				STACKING GEL
	7.5%	10%	12%	15%	4%
30% Acry-Bis	4.3 mL	5 mL	6 mL	7.5 mL	2 mL
Tris-HCl 0,5M pH=6,8	-	-	-	-	1.5 mL
Tris-HCl 2M pH=8.8	3.4 mL	3 mL	3 mL	3 mL	-
Agua MilliQ	10.15 mL	7 mL	6 mL	4.5 mL	1.5 mL
20% SDS	85 µL	75 µL	75 µL	75 µL	75 µL
10% APS	85 µL	75 µL	75 µL	75 µL	125 µL
TEMED	5.64 µL	5 µL	5 µL	5 µL	12.5 µL

When the running gel polymerized, the water was removed. At the same time, the stacking gel solution was prepared and the rest of the plate was filled with it. Then, the comb was placed to form the wells. Once the gel polymerized it was ready to use.

Table 12. Reagents used for casting the gels and their functions.

Reagent	Function
Acrylamide	The monomers that polymerize forming the gel matrix
Bisacrylamide	Cross-linking reagent to form polyacrylamide
APS	Polymerization initiator
TEMED	Polymerization catalyst
Tris ($C_4H_{11}NO_3$)	Solvent for preparing the gels
SDS	Denatures and negatively charges the proteins

The gels were run under reducing conditions by the addition of DTT to the samples. In fact, it ensures the denaturation of the proteins, since it cleaves the sulfide bonds of the chains and disrupts the tertiary and quaternary structure of the macromolecules. Therefore, under these conditions, the migration speed of the proteins is proportional to their molecular mass, and not due to their charge or 3D structure. Before adding the samples to the gel, 40 µg of each sample was mixed with Laemmli 5X loading buffer (60 mM Tris-HCl at pH 6.8, 2% w/v SDS, 10% glycerol, 0.002% bromophenol blue) and also with DTT reducing agent at 1 mM. In addition, RIPA buffer was added up to 25 µL, so that all the samples contained 40 µg of protein in a volume of 25 µL. Then, the samples were heated at 37 ºC for 30 minutes, since the proteins of interest were mainly membrane proteins and they tend to aggregate if denatured at 95 ºC[150].

Table 13. Loading buffer composition.

Reagent	Function
Bromophenol blue	Monitor the migration of the samples through the gel
Glycerol	Increases the density of the sample so that it layers in the well and does not get mixed with the running buffer
DTT	Break the disulfide bonds and disrupt the tertiary and quaternary structure of proteins
SDS	Denatures the proteins and gives them overall negative charge

At the same time, the gels were assembled in the electrophoresis tank and the inner chamber between the gels was filled with running buffer (25 mM Tris pH 8.3, 190 mM Glycine, 0.1% SDS). When the samples were denatured, the comb was taken out and 20 µL of each sample was loaded inside each well. The first well was left empty for the molecular weight marker to estimate the mass of the proteins of interest that were resolved in the gel. When all the wells were filled, the inner chamber and the bottom of the outer chamber were filled with running buffer. Then, the electrophoresis was run at 120V for the first 10 minutes to concentrate all the proteins in the stacking gel. After this time, the voltage was set at 180 V for 1 hour to separate the proteins.

- Transfer:

After separating the proteins throughout the gel, they were transferred to a solid support membrane of nitrocellulose for immunodetection with specific antibodies. Based on the same principle as electrophoresis, in this case, a horizontal electric field was applied, so that the separated proteins were immobilized in the same migration position as in the gel. For this, the membrane and the gel were assembled inside a paper and sponge sandwich, and when voltage was applied, the proteins migrated from the gel towards the anode, where the membrane was located. The wet transfer method was employed, so all the sandwich elements (paper, sponge, gel, membrane) were completely immersed in transfer buffer before assembly. This method is recommended for larger proteins and higher quality blots are achieved. However, it is a slow approach and the system is heated, so an ice bucket was introduced into the tank, which was placed in a tray surrounded by ice.

After constructing the sandwich, it was introduced into the tank, which was filled with transfer buffer (25 mM Tris pH 8.3, 190 mM Glycine, 0.1% SDS, 5% Methanol) and an ice bucket. The transference was carried out for 3 hours at 300 mA.

Once it finished, the membrane was stained with Red Ponceau solution (0.1% of Red Ponceau, 1% of glacial acetic acid) to confirm the correct transfer of proteins, since this solution stains all the protein bands that have been immobilized in the membrane. Then, the membrane was rinsed with TBS until the staining was removed.

- Immunoblotting:

To avoid non-specific binding of antibodies to the membrane, membrane spaces not occupied by transferred proteins were blocked, using a blocking reagent with higher affinity for the membrane than the antibodies used afterwards. In this case, 5% of non-fat milk diluted in 0.1% TBS-Tween 20 (TBS-T) was used under stirring at room temperature for 1 hour. After this, the membrane and the primary antibody diluted in the blocking reagent at the recommended concentration **(Table 14)** were incubated overnight at 4 ºC under agitation.

Table 14. Primary antibodies used for western blot.

Primary antibody	Reference	Company	Origin	Dilution
PLD2	#AO2358a	Abgent	Mouse	1/1500
EGFR	Ab52894	Abcam	Rabbit	1/2000
PKCα	Sc-8393	Santa Cruz	Mouse	1/1000
PLD1	#3832	Cell Signaling	Rabbit	1/1000
PLA_a	Sc-376563	Santa Cruz	Mouse	1/1000
PLC	Sc-5291	Santa Cruz	Mouse	1/1000
α-Tubulin	T9026	Sigma Aldrich	Mouse	1/3000

After this first incubation, the primary antibody was discarded and the membrane was washed three times for 5 minutes with TBS-T. Next, the secondary antibody diluted **(Table 15)** in blocking reagent was incubated with the membrane for 2 hours at room temperature. This is a species-specific antibody directed against the constant region of the primary antibody and is labeled with HRP (horseradish peroxidase).

Table 15. Secondary antibodies used for western blot.

Secondary antibody	Reference	Company	Dilution
Anti-Mouse IgGκ-HRP	Sc-516102	Santa Cruz	1/5000
Anti-Mouse IgG-HRP	1032-05	Southern Biotech	1/8000
Anti-Rabbit IgG-HRP	Ab102279	Abcam	1/10,000

- Detection:

The HRP enzyme conjugated to the secondary antibody generates chemiluminescence when incubated with ECL solution (Enhanced ChemiLuminescence). The enzyme catalyzes the oxidation of the luminol present in the ECL, resulting in the emission of light. Using a charge-couple device camera-based imager, digital images of the chemiluminescence of the membrane were captured using the Gene Snap program.

- Image analysis:

The band intensity analysis for each protein detected was performed by determining the densitometry of each lane with Image J program. The intensity of each lane was normalized to the relative amount of the control protein –tubulin- of each sample.

2.5.2. Immunofluorescence

This widely used approach allows the localization and the determination of the expression levels of a given protein. This methodology can be used in both tissue sections and cultured cells. This immunostaining technique is based on tagging antigens by using fluorescent molecules such as Hoechst to stain the DNA or specific antibodies labeled with fluorochromes. These molecules absorb ultraviolet light at a certain wavelength, but emit light at a higher wavelength, which is captured by specific filters of a fluorescence microscope. Here indirect immunofluorescence was used. Thus, an unlabeled antibody specifically detected the antigen of interest. Then, a species-specific fluorescent-labeled secondary antibody was bound to the primary antibody and this sandwich was detected using a fluorescence microscope.

In this study, cultured cells were analyzed. For this, the cells were seeded in round coverslips assembled inside the wells of 24-well plates. In each coverslip, 500 µL of cells were incubated

overnight to allow their adhesion to the immunostaining support. Then, the cell medium was removed and the cells were washed with PBS. For the immobilization of the antigens, the cells were fixed with 4% of paraformaldehyde for 15 minutes at room temperature. After washing the cells three times with PBS, they were permeabilized for 5 minutes with 0.1% triton-X100 in PBS to ensure the entry of the antibody to its antigen. At that time, the cells were rinsed abundantly with PBS, and all nonspecific binding sites were blocked using 5% FBS diluted in PBS for 1 hour at room temperature. Then, the blocking solution was discarded and the primary antibody was diluted in blocking solution for an overnight incubation at 4 ºC. After washing the cells with PBS to remove the unbound primary antibody, the secondary antibody tagged with the fluorescence dye was added for 2 hours in the dark. Finally, the cells were washed and Hoechst was added for 5 minutes at a concentration of 1 µg/mL to stain the nucleus of the cells. Immediately, coverslips were mounted with Fluoromount-G on a microscope slide. Then, the samples were visualized in a fluorescence microscope.

2.6. PLD enzymatic activity assay *in vitro*

This assay was carried out in the laboratory of Dr. Julian Gomez-Cambronero in Wright State University (OH, USA) following their own protocol[151]. Under normal conditions, PLD enzyme cleaves the phospholipids of the biological membranes, releasing phosphatidic acid and the polar group of the phospholipid. However, in the presence of a primary alcohol such as butanol, PLD follows a specific reaction called transphosphatidylation, in which phosphatidylbutanol is generated as an end-product.

Before starting the experiment, cellular sonicates were prepared. The stored cell pellets were re-suspended in a special lysis buffer (5 mM Hepes, pH 7.8, 100 µM Na orthovanadate, 0.4% Triton X-100) plus Aprotinin (2 µg/mL) and Leupeptin (5 µg/mL) protease inhibitors. Keeping the samples on ice, they were sonicated for 10 seconds. Next, BCA assay was carried out for protein quantification and 50 µg were processed for the measurement of PLD activity.

First, liposomes containing PC(8:0/8:0) and PIP_2 (Phosphatidylinositol 4,5-bisphosphate), which is a cofactor of PLD, were prepared. Then, [^{3}H]butanol and the liposomes were mixed. The final concentration of all the reagents in the assay mix was: 45 mM HEPES pH 7.8, 150 nM NaCl, 0.16% Triton X-100, 3.5 mM PC(8:0/8:0), 1 µM PIP_2, 0.9 µCi [^{3}H]butanol. This reaction mixture was incubated together with the cell sonicates at 30 ºC for 20 minutes in a shaker incubator to allow the enzyme act. At this point, the reaction was stopped using an ice-cold stopping solution (2 MeOH/ 1 Cl_3CH/ 10 N-HCl), Cl_3CH and H_2O. After vortexing all the tubes 3 times, they were centrifuged at 14,000 g for 30 seconds, and a biphasic separation was achieved: in the upper phase there was an aqueous phase containing the unincorporated [^{3}H]butanol; separated by a white meniscus was the chloroform phase where all the lipids were dissolved. Forthwith, the aqueous phase was aspirated and the meniscus discarded, while the lipid phase was transferred to new clean tubes, which were tightly capped to avoid evaporation.

At this point, the chromatography solvents and the TLC plate were prepared. For this, in a separatory funnel, 260 mL ethyl acetate, 40 mL iso-octane, 60 mL acetic acid and 200 mL H_2O were stirred and let to stand to allow phase separation. Once proper separation was achieved, the lower phase was discarded and the upper phase was poured into the TLC chamber. At the same time, the TLC plate was activated by heating it at 115 ºC for 15 minutes.

After drying the samples under a N_2 stream, they were reconstituted in 37 µL of Cl_3CH/MeOH (9:1) and loaded on the TLC plate. On both side-lanes of the plate, phosphoethanol and phosphobuthanol standards were spotted respectively, so they run at the same time as the study samples. When the chromatography was stopped, the radiolabeled phosphobutanol from the study samples would had run in the area between both standards. Therefore, in each lane, the area between both standards was scraped and introduced into a scintillation vial together with scintillation cocktail. Eventually, the radioactivity of each sample was determined in a scintillation counter.

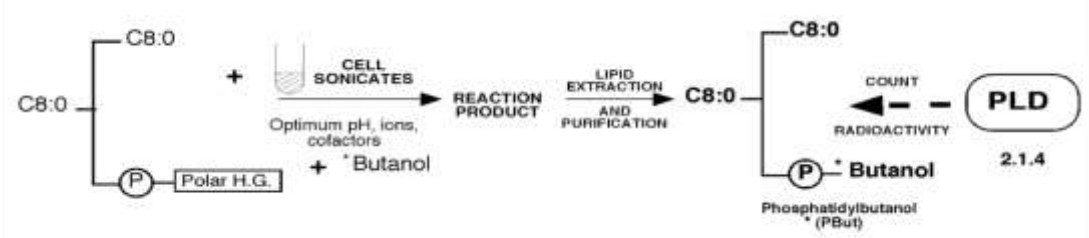


Figure 26. Schematic representation of the PLD enzymatic assay reaction[149].

RESULTS

1. Lipidomic analysis of lipid extracts of melanocyte and melanoma cell lines

The UHPLC-ESI-MS/MS methodology used in this study enabled the identification of the lipidome of normal skin melanocytes, nevus derived melanocytes, and primary and metastatic melanoma cell lines, in order to compare them and determine if the differential lipid fingerprint could discriminate the study groups. For this, the lipids of 2 different passages of 19 cell lines were extracted using the Bligh & Dyer method, followed by an UHPLC-ESI-MS/MS approach, as described in the Materials and Methods section. Concretely, three different normal skin melanocytes, five melanocytes cell lines isolated from nevus, five primary, and six metastatic melanoma cell lines were analyzed **(Table 16)**. Four lymph node metastatic cell lines and two subcutaneous metastatic cell lines made up the latter group.

Table 16. Pathological classification of the cell lines studied by UHPLC-ESI-MS/MS methodology.

Skin melanocytes	Nevus	Primary Melanoma	Metastatic Melanoma	
			Lymph node	Subcutaneous
HEMn-LP	N1	A375	RPMI7951	Colo-800
HEMn-MP	N2	Mel-Ho	Hs294T	HT-144
HEMn-DP	N3	G-361	A2058	
	N4	Sk-Mel-28	Sk-Mel-3	
	N6	Sk-Mel-31		

This analytical approach allowed the identification of a total of 213 different lipid species within the mass range of 50 to 1200 units **(Appendix, Table 39)**. These lipid molecules included 93 glycerophospholipids, 59 glycerolipids, 31 sphingolipids, 6 cholesterol esters and 24 free fatty acids. In particular, 95 species of eight different subclasses were identified in the negative ESI ionization method (PE, ether-PE, Lyso-PE, PI, PS, PG, Cer, and FFA), and 118 species of nine distinct subclasses were characterized (PC, ether-PC, Lyso-PC, SM, Hex-Cer, Cer, TG, DG, and CE) in the positive ionization mode **(Fig. 27)**.

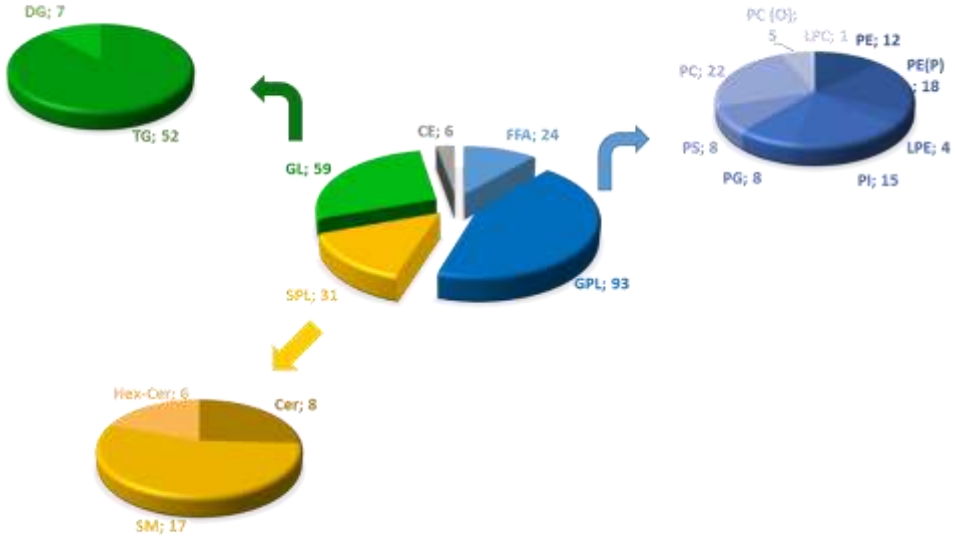


Figure 27. Categorization of the lipid species identified in the UHPLC-ESI-MS/MS method. In the central pie chart, the number of species identified in each lipid class is represented. In the contiguous graphs, the distribution among the subclasses of each lipid specie detected in each class are presented.

1.1. Differential global lipotype between melanocytes and melanoma cells

A non-supervised principal components analysis (PCA) was conducted to overview the relationship between the samples and their lipid profile. This analysis was performed to visualize the trends of the samples, and determine if there is any natural aggrupation of the results. The PCA demonstrated the good quality and robustness of the data obtained, as all the QC samples were grouped together in both ESI+ and ESI-, no matter the moment of the injection (at the beginning, along the experiment, or at the end) **(Fig. 28)**.

However, a strong outlier was detected in both ionization modes. This was one of the duplicates of HT144 cell line, which belongs to the subcutaneous metastatic melanoma (MMNS) group. As it can be seen in **Figure 28**, this sample lied outside the ellipse and the DModX plot that represents the 95% confidence interval set by Hotelling's T2. Thus, it was removed from the analysis, as well as the other samples of the MMNS group, since there were only three more samples in the group, not enough for a good representation of this type of metastasis. Consequently, a new PCA analysis was conducted after excluding these samples.

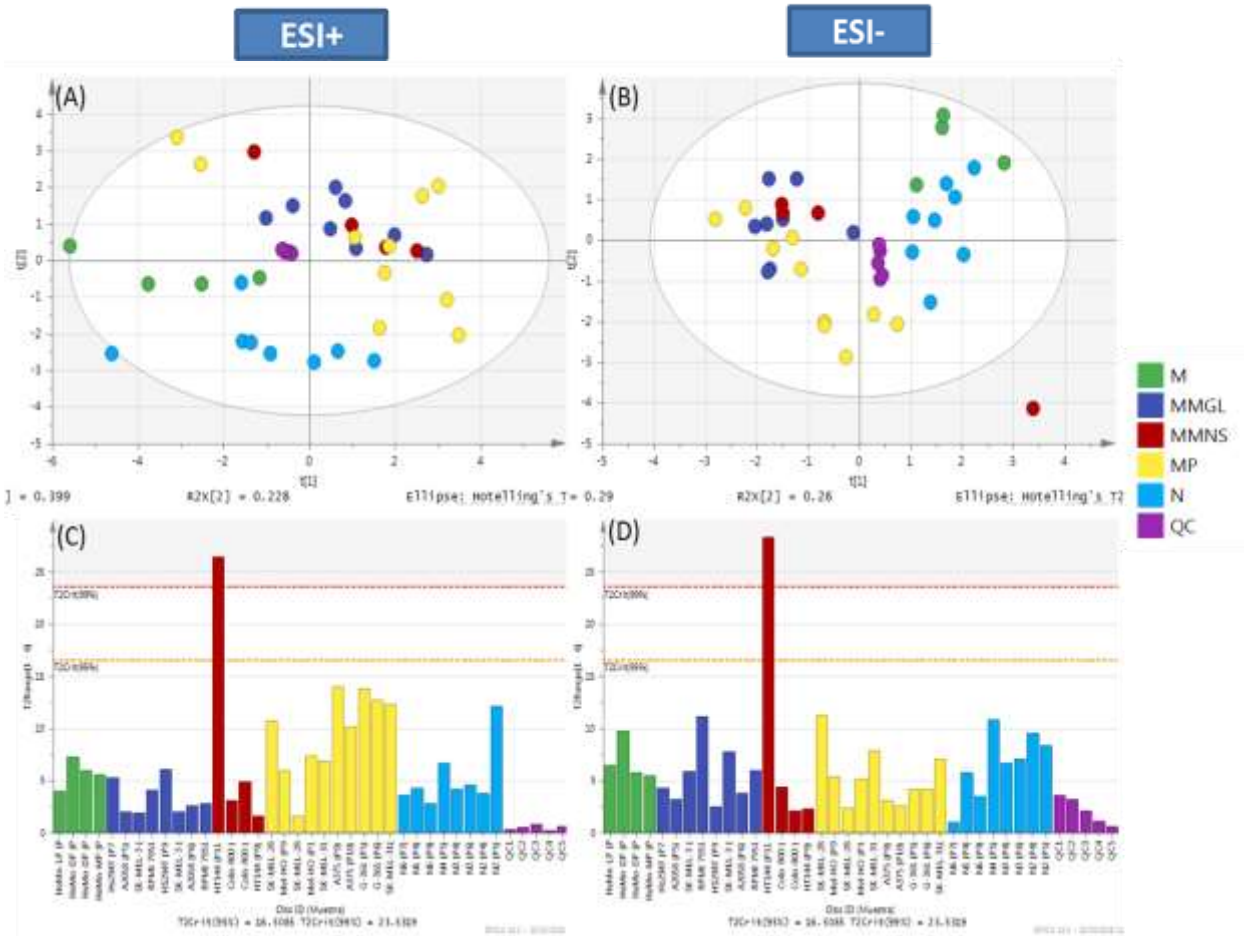


Figure 28. PCA of the study samples and QC samples in both ESI + and ESI-. Representation of the score plot of PCA analysis and its respective Hotelling's T2 plot in ESI+ (A/C) and ESI- (B/D). Legend: (M) Melanocytes: green; (N) Nevus: blue; (MP) Primary Melanoma: yellow; (MMGL) Lymph node Metastatic Melanoma: blue; (MMNS) Subcutaneous Metastatic melanoma: red; (QC) Quality controls: purple.

In the new analysis, the distance to the model in X-space (DModX) plots suggested that N4 (passage 5) in ESI+ and Hs294t (passage 9) in ESI- were moderate outliers, as these samples lied outside the maximum tolerable distance (DCrit=0.05). However, as they were not represented outside the ellipse in the score scatter plot, they were maintained in the analysis.

An overview of the lipidomic analysis by PCA demonstrated that the lipid species detected in this study were sufficient to classify the samples in non-malignant cells (melanocytes (M), nevus (N)) and malignant cell lines (primary (MP) and lymph node metastatic melanoma (MMGL)) in both ESI+ and ESI- **(Figure 29)**. Nonetheless, an overlap between nevus and skin melanocytes, and between primary and metastatic melanoma groups can also be observed, thus, this model was able to differentially cluster normal and malignant samples, but fails to classify them according to the progression of the disease. The score plot of PC1 vs PC2 in ESI+ revealed R2=0.830, Q2=0.736, and R2=0.884, Q2=0.696 in ESI-. Although R2 and Q2 results were not excellent, they indicate that the model fit is good and has a good prediction ability, thus the aggrupation of the samples is not by chance.

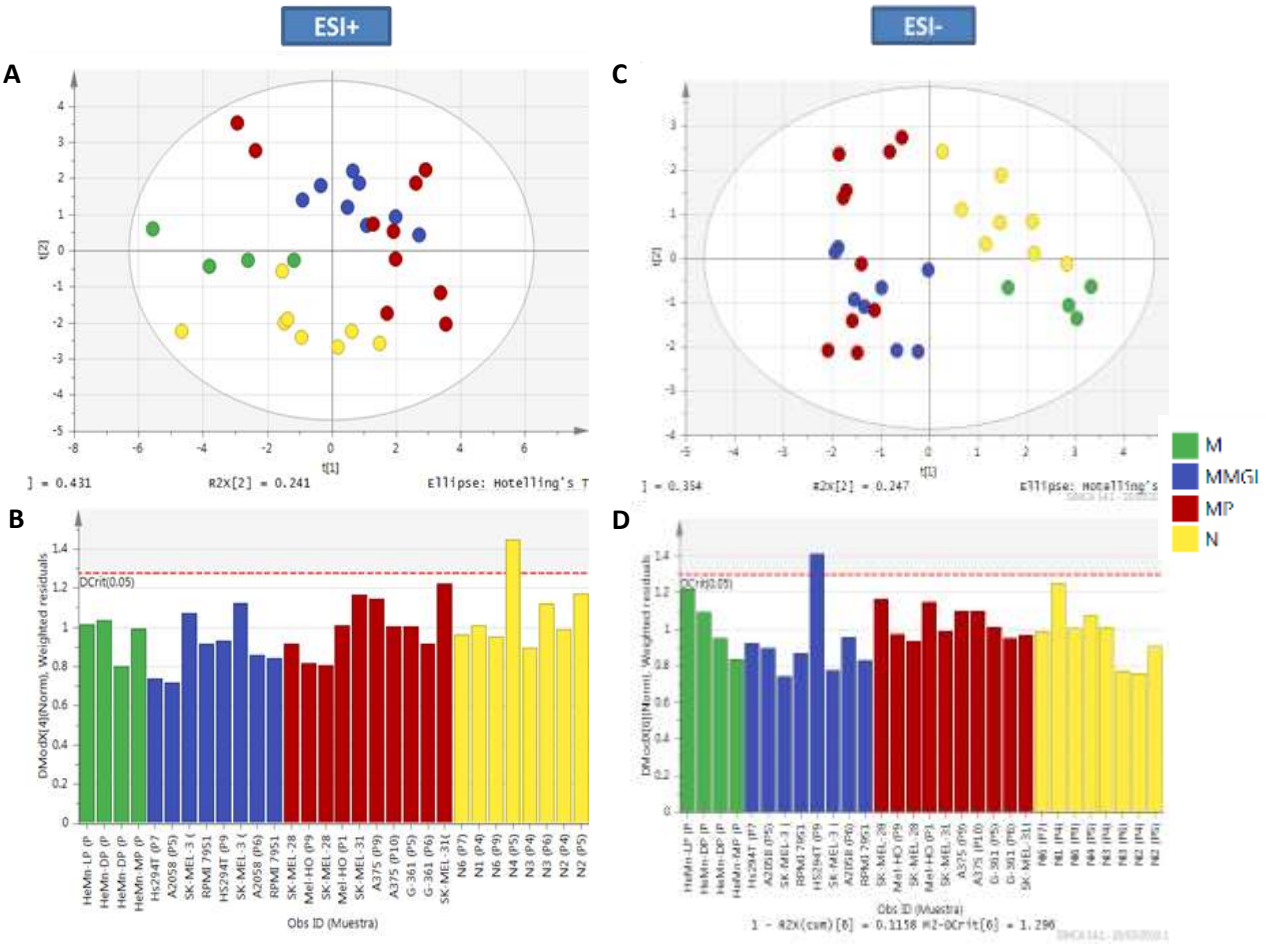


Figure 29. Principal components analysis of the samples in ESI+ and ESI-. A/C) Score scatter plot representing the PCA model and B/D) their corresponding DModX plot. Legend: (M) Melanocytes: green; (N) Nevus: yellow; (MP) Primary Melanoma: red; (MMGL) Lymph node Metastatic Melanoma: blue.

Next, several supervised analyses were performed. First, as the PCA analysis did not achieve the total separation of the four study groups, PLS-DA (partial least square discriminant analysis) was carried out. In this study, the principal components of each study group were compared in order to build a classification model that maximizes the differences between the groups. In this way, the samples were classified according to their lipotype. In the graphs of **Figure 30 & 31**, various two to two comparisons of the groups were performed. Here, a clear separation of normal and pathological cell lines was achieved, confirming a differential lipid pattern between these groups in both ESI+ and ESI-. Indeed, for ESI+ results **(Fig. 30)**, the score plots of the comparisons nevus vs metastatic melanoma **(A)** (R2=0.982, Q2=0.966), nevus vs primary melanoma **(B)** (R2=0.976, Q2=0.932), and non-malignant (M+N) vs malignant (MP+MMGL) **(D)** (R2=0.963, Q2=0.898) revealed that the lipid profiles of these groups are pronouncedly different. Conversely, as it can be seen in the score plot of the comparison skin melanocytes vs nevus melanocytes **(C)** (R2=0.831, Q2=0.594), the separation between these cell lines is not so clear. Similar results were achieved in ESI- ionization mode **(Fig. 31)**. Specifically, the score plots revealed a different lipid phenotype in the comparisons of metastatic melanoma vs nevus **(A)** (R2=0.956, Q2=0.933), nevus vs primary melanoma **(B)** (R2=0.967, Q2=0.887), non-malignant (M+N) vs malignant cells (MP+MMGL) **(E)** (R2=0.924, Q2=0.871). However, the lipid phenotype of skin vs nevus melanocytes **(C)** (R2=0.689, Q2=0.433), and primary vs metastatic melanoma cells **(D)** (R2=0.796, Q2=0.515) were not different enough to classify the samples correctly with this analysis.

To validate this model, 999 permutation tests were carried out, by permutating the variable Y in the two studied groups. For this, the cumulative value of R2 and Q2 of 999 random rearrangements of the Y variables were performed, and then the reconstruction of the PLS-DA model was conducted. The negative intersection of the Q2Y line of tendency obtained for all the models demonstrated that the PLS-DA analyzes adjust well the data and that the classification is not by chance, as the permutated data on the left was lower than the original data on the right **(Appendix, Figure 60 & 61)**.

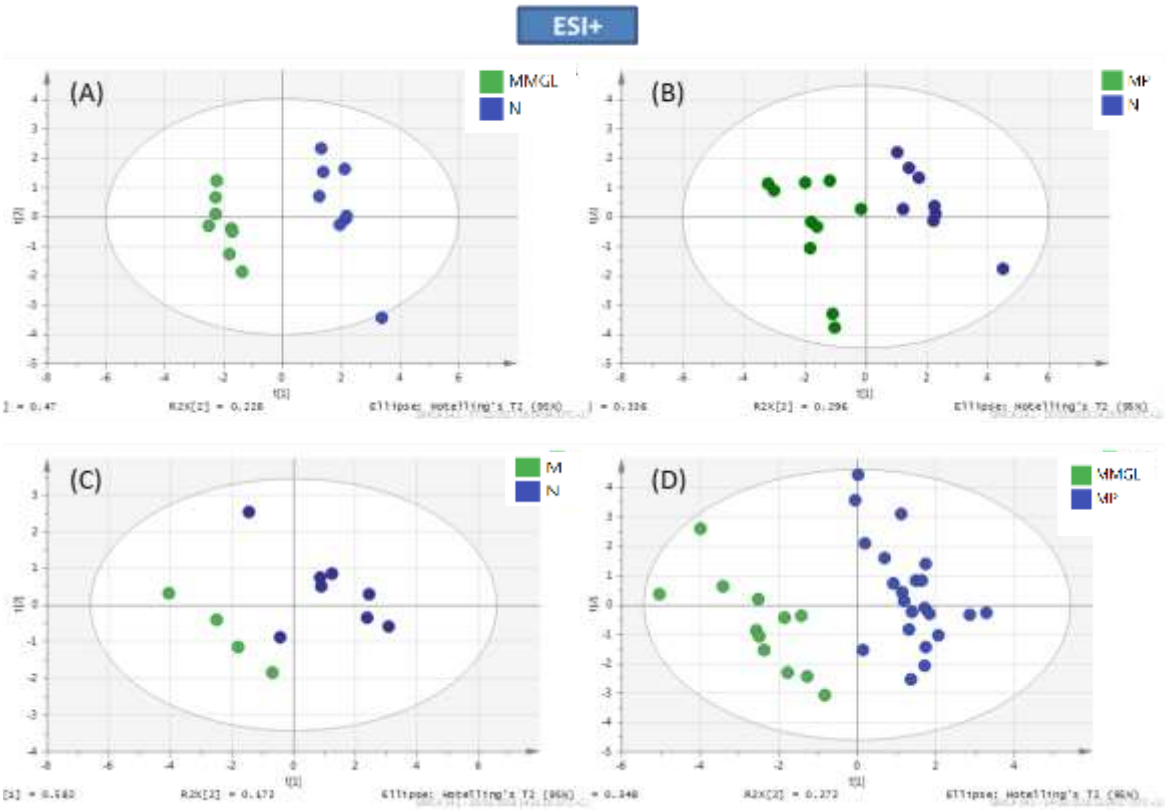


Figure 30. PLS-DA analysis of the samples detected in ESI+ classified into groups. (A) Metastatic melanoma (green) vs nevus (blue); (B) Primary melanoma (green) vs nevus (blue); (C) Melanocytes (green) vs nevus (blue); (D) skin + nevus melanocytes (green) vs primary + metastatic melanoma (blue).

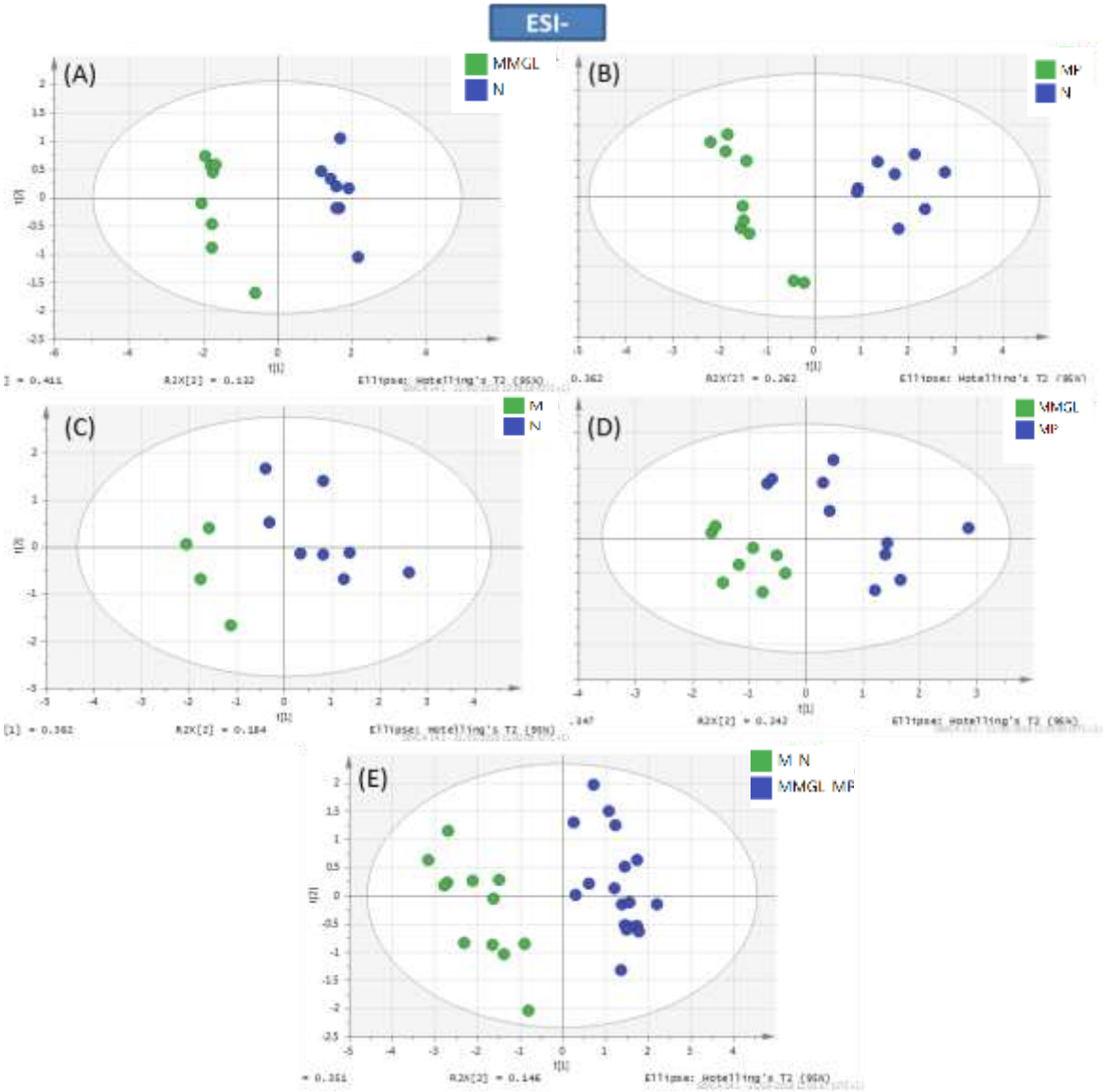


Figure 31. PLS-DA analysis of the samples detected in ESI- classified into groups. (A) Metastatic melanoma (green) vs nevus (blue); (B) Primary melanoma (green) vs nevus (blue); (C) Melanocytes (green) vs nevus (blue); (D) Metastatic melanoma (green) vs Primary melanoma (blue); (E) skin + nevus melanocytes (green) vs primary + metastatic melanoma (blue).

In addition, a second validation test was performed using ANOVA analysis. As it can be seen in the **table 17**, all the models have p-values < 0.05, confirming the validity of the models, except for the M vs N comparisons in both ESI+ and ESI-. Therefore, the R2, Q2, permutation Q2 and p-values indicate that the stablished PLS-DA models can accurately and reliably differentiate the cell lines studied based on the lipid fingerprint, except for the lipid phenotype of melanocytes and nevus that are not distinct enough to be differentiated by this approach.

Table 17. Validation of the PLS-DA models. The validity of the models constructed by PLS-DA analyses was stablished by Q2 obtained in the permutation tests and the p-values in the ANOVA analysis.

Models	Ionization mode	PCs	R2	Q2	p	Permutation Q2
MMGL vs N	ESI+	2	0.982	0.966	$1.27x10^{-7}$	-0.302
MP vs N		3	0.976	0.932	$3.16x10^{-5}$	-0.480
M vs N		2	0.831	0.594	0.11	-0.226
M+N vs MMGL+MP		4	0.963	0.898	$8.25x10^{-11}$	-0.476
MMGL vs N	ESI-	1	0.956	0.933	$2.29x10^{-8}$	-0.219
MP vs N		3	0.967	0.887	$2.11x10^{-4}$	-0.443
M vs N		1	0.689	0.433	0.33	-0.226
MMGL vs MP		2	0.796	0.515	0.048	-0.254
M+N vs MMGL+MP		2	0.924	0.871	$8.60x10^{-11}$	-0.312

1.2. Different lipid classes exhibit altered levels in melanoma cells

The results of the previous section confirm that normal melanocytes and malignant melanoma cells have a different lipid profile. A deeper view of the data obtained demonstrates that many of the lipid molecular species detected do not present the same intensity in the four study groups.

1.2.1. Glycerophospholipids

93 different glycerophospholipid species were identified in this work **(Appendix, Table 40)**. They were distributed in eight different subclasses, namely, phosphatidylethanolamines (with 12 molecular variants), ethanolamine ether lipids (18), lyso-phosphatidylethanolamines (4), phosphatidylcholines (22), choline ether lipids (5), lyso-phosphatidylcholine (1), phosphatidylinositol (15), phosphatidylglycerols (8) and phosphatidylserines (8). In our study, we have identified a tendency of increased intensity for glycerophospholipid species in metastatic melanoma cells, which are highly proliferative and therefore require higher levels of these lipids.

Table 18. Sum of the intensities obtained for all lipid species that make up each lipid subclass and comparison of the intensity variation of each subclass in the different study groups, related to control group (Melanocytes [M]=1). Legend: (M) Skin melanocytes; green; (N) nevus melanocytes: yellow; (MP) primary melanoma: red; (MM) metastatic melanoma: blue.

	Skin melanocytes (M)		Nevus melanocytes (N)		Primary Melanoma (MP)		Metastatic Melanoma (MM)	
	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M
Ethn. lipids	709,499	1.00	438,415	0.62	426,026	0.60	535,813	0.76
PE (P/O)	342,846	1.00	226,808	0.66	195,850	0.57	276,641	0.81
PE	346,236	1.00	204,261	0.59	220,082	0.64	248,179	0.72
LPE	20,417	1.00	7,346	0.36	10,094	0.49	10,993	0.54
Choli. lipids	7,068,929	1.00	4,846,827	0.69	6,424,730	0.91	9,000,850	1.27
PC (P/O)	210,327	1.00	51,091	0.24	629,584	2.99	824,430	3.92
PC	6,857,370	1.00	4,782,295	0.70	5,793,932	0.84	8,175,242	1.19
LPC	1,232	1.00	628	0.51	1,214	0.99	1,178	0.96
PI	239,144	1.00	149,704	0.63	166,719	0.70	272,480	1.14
PG	39,980	1.00	25,981	0.65	21,528	0.54	40,818	1.02
PS	195,372	1.00	136,855	0.70	99,014	0.51	122,838	0.63

Among all the GPL subclasses, lipid molecules with ethanolamine head group present the greatest structural variability, since 34 species have been identified. In particular, 18 ethanolamine ether lipids, 12 phosphatidylethanolamine and 4 lyso-phosphatidylethanolamine molecular species have been detected. The comparison of the intensities detected for ethanolamine bearing lipids in the four study groups suggests that there is an increased presence of these lipids in normal skin melanocytes, whereas nevus melanocytes and melanoma cells showed a decreased presence **(Fig. 32 (A))**.

Regarding PE ether species, the intensity of eight (M), seven (N), or six (MP & MMGL) of the 18 species identified in each group represent 5% or more of all the intensities accounted for the subclass in each study group, making up the 87-82% of the sum of all the intensities detected for the subclass. The remaining variants identified for this subclass are low abundant, however, they might have an important contribution to cell function. PE(P-16:0/18:1) is the most abundant molecule in skin melanocytes (20%), primary melanoma (35%) and metastatic melanoma (28%), but PE(P-38:4) has the highest intensity levels in nevus cells (20%) **(Appendix, Table 40)**. The overall comparison in this subclass shows a tendency of decreased expression of PE ether lipids in nevus (0.66 related to skin melanocytes), primary (0.57) and metastatic melanoma (0.81) **(Table 18, Figure 32(A))**. However, there is not a common tendency among all the species of the subclass, since some of the lipids increase their intensity accordingly to the progression of the malignancy, whereas others reduce it.

The PE subclass is composed by 12 different species, and among them, PE(18:0/18:1) is maintained as the most abundant in the four study groups. According to the principal lipids of the subclass, with an intensity higher than 5% of all the lipids detected in each group, there are eight in skin melanocytes accounting for by 87% of the total and seven in nevus representing the 82%. Likewise, the six principal species are common to primary and metastatic melanoma standing for the 84% **(Appendix, Table 40)**. Moreover, all the groups share five of them. In general, there is a decreased expression of these lipids from normal melanocytes to the rest of the cell groups **(Table 18, Figure 32(A))**.

A sharp decrease in the intensity of lyso-phosphatidylethanolamine lipids is observed in nevus melanocytes and melanoma cells, although there is great variation among the cell lines that make up each study group.

Representing the choline containing GPLs there are 22 phosphatidylcholines, 5 ether lipids and 1 lyso-phosphatidylcholine. A general comparison of all these molecules demonstrates an increased presence of choline lipids in metastatic melanoma (1.27) compared to the other three groups **(Table 18, Fig. 32 (B))**.

PCs in particular, have PC(16:0/18:1) as the most abundant specie. Skin and nevus melanocytes, as well as metastatic melanoma cells have six principal molecules that make up 85%, 84% and 89% of the total intensities obtained for each study group, respectively. However, primary melanoma cell lines have seven principal lipids that represent the 92% of all **(Appendix, Table 40)**. A global comparison of the intensities identified for the PCs shows a tendency of increased abundance of PCs in metastatic cell lines (1.19) compared to the other study groups **(Table 18, Figure 32(B))**.

Although only five choline ether-lipids have been detected, all of them show an increase in their intensity that correlates with the progression of melanoma. The fatty acid side-chains of the most abundant FAs in this lipid subclass coincide with the most abundant PC-FA, namely PC(O-16:0/18:1).

Table 19. Sum of the intensities obtained for all the PC, PC(P/O), PE and PE(P/O) lipid species detected, and the ratios PC/PC(P/O), PE/PE(P/O), choline lipids/ethanolamine lipids, PC/PE, PC(P/O)/PE(P/O), LPC/LPE. The results of each study group are compared to the values obtained for skin melanocytes, that is considered the control group (Melanocytes [M]=1). Legend: (M) Skin melanocytes: green; (N) nevus melanocytes: yellow; (MP) primary melanoma: red; (MM) metastatic melanoma: blue.

	Skin melanocytes (M)		Nevus melanocytes (N)		Primary Melanoma (MP)		Metastatic Melanoma (MM)	
	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M
PC (P/O)	210,327	1.00	51,091	0.24	629,584	2.99	824,430	3.92
PC	6,857,370	1.00	4,782,295	0.70	5,793,932	0.84	8,175,242	1.19
PC/PC(P/O)	**32.60**	**1.00**	**93.60**	**2.87**	**9.20**	**0.28**	**9.92**	**0.30**
PE (P/O)	342,846	1.00	226,808	0.66	195,850	0.57	276,641	0.81
PE	346,236	1.00	204,261	0.59	220,082	0.64	248,179	0.72
PE/PE(P/O)	**1.01**	**1.00**	**0.90**	**0.89**	**1.12**	**1.11**	**0.88**	**0.89**
Chl/Eth lip.	**9.96**	**1.00**	**11.03**	**1.11**	**15.08**	**1.51**	**16.80**	**1.69**
PC/PE	19.81	1.00	23.41	1.18	26.33	1.33	3.,94	1.66
PC(P/O) / PE(P/O)	0.61	1.00	0.23	0.37	3.21	5.24	2.98	4.86
LPC/LPE	0.06	1.00	0.09	1.42	0.12	2.00	0.11	1.78

A common feature observed in many cancers is the decreased ratio of PC/PC(P/O) or PE/PE(P/O). This trend has also been observed in our results. For choline lipids in particular, there is a high decrease of the PC/choline ether lipids ratio in metastatic and primary melanoma compared to melanocytes **(Table 19)**. Conversely, there is a high increase in this ratio for nevus melanocytes compared to skin melanocytes. Interestingly, a 3-fold and 10-fold decrease has been observed in malignant cells vs skin and nevus melanocytes, respectively, while this ratio is maintained similar in primary and metastatic melanoma, giving a hint of the importance of ether lipids and peroxisomes in melanoma. However, no alteration has been detected in ethanolamine containing lipids.

It is widely known that choline and ethanolamine containing glycerophospholipids are major constituents of cell membranes. The balance between these two lipid subclasses modify the biophysical properties of the bilayers. In our study, the choline/ethanolamine species ratio is higher in both primary and metastatic melanomas compared to melanocytes **(Table 19, Fig. 32 (C))**. This tendency is observed for PCs/PEs and lyso-PCs/lyso-PEs, being significantly higher in both primary and metastatic melanomas when comparing choline and ethanolamine ether lipids. Markedly, there is a 5-fold increase in primary and metastatic melanomas vs skin melanocytes and a 15-fold increase vs nevus, giving insight into the importance of these lipids in the development and metastatic process of melanoma.

Figure 32. Comparison of the sum of the intensities of all the ethanolamine lipid species (A) and choline bearing lipid (B) species in the four study groups. (C) Ratio of the intensities of choline/ethanolamine lipid species in the four study groups. All the sums are related to skin melanocyte values (M = 1). A) Analysis of the sum of the intensities of all the lipids that have ethanolamine head group itemized in ethanolamine ether lipids (PE(P/O), phosphatidylethanolamines (PE) and lyso-phosphatidylethanolamines (LPE). B) Sum of the intensities of all glycerophospholipids with choline head group, distributed in choline ether lipids (PC(P/O) and phosphatidylcholines (PC). C) Analysis of the ratio of the intensities of all ethanolamine / choline lipids, PC/PE ether lipids, PC/PE glycerophospholipids and lyso-PC/lyso-PE lipids. Legend: skin melanocytes: green; nevus melanocytes: yellow; primary melanoma: red; metastatic melanoma: blue.

Fifteen different phosphatidylinositol molecules have been detected with this methodological approach. In normal tissues PI(18:0/20:4) is the most abundant molecule of this subclass, a feature that has also been observed in our study. Indeed, this is the principal PI in the four study groups, accounting for the 21% of all the PI intensities detected in skin melanocytes, 26% in nevus melanocytes, 18% in primary melanoma, and 17% in metastatic melanoma **(Appendix, Table 40)**. The global comparison of the intensities obtained for these lipids establishes a tendency of decreased relative abundance in nevus (0.63 normalized to skin melanocytes) and primary melanoma (0.70), with a slight increase in metastatic melanoma (1.14) **(Table 18, Fig. 33)**. However, the lipid extraction method used here hampers the extraction of phosphorylated PIs, which play a very important role in cancer signaling. Furthermore, there is great variance in the intensities of these lipids within the skin melanocytes cell lines, being cell line-specific. Therefore, further experiments with specific extraction and analytical methods are needed to confirm the relative abundance of these molecules in melanoma.

Phosphatidylglycerols have been found to have a lower relative abundance in nevus melanocytes (0.65) and primary melanoma (0.54), while it is very similar in skin melanocytes (1.00) and metastatic melanoma (1.02) **(Table 18, Fig. 33)**. The intensity detected for the individual species of this subclass varies within each study groups, thus, it depends on the particular cell line. In fact, PG(16:0/18:0) or PG(18:0/16:0) are the dominant species in skin melanocytes (48%), nevus cells (41%) and primary melanomas (28%), whereas it decreases to 24% in metastatic melanoma **(Appendix, Table 40)**. Conversely, the principal lipid of the latter group is PG(18:0/16:1) or PG(18:1/16:0) representing 25% of all the PGs detected in metastatic melanomas. Interestingly, this molecule might be important for melanoma cells function since its presence in melanocytes goes from 6-8% of all the PGs detected to 20% and 25% in primary and metastatic melanoma, respectively.

Concerning the last glycerophospholipid subclass identified, phosphatidylserines, there is a tendency to decrease their presence in melanoma **(Table 18, Fig. 33)**. In this subclass, the most abundant specie is PS(18:1/18:0) and/or PS(18:0/18:1), which is common to all the study groups and represents about half of the intensities detected for all the lipid molecules that make up this subclass.

Figure 33. Comparison of the sum of the intensities detected for all the phosphatidylinositol species (PI), phosphatidylglycerol species (PG) and phosphatidylserine species (PS) in the four study groups, related to skin melanocyte values (M=1). Legend: skin melanocytes: green; nevus melanocytes; yellow; primary melanoma: red; metastatic melanoma: blue.

1.2.2. Glycerolipids

Triglycerides are the lipid class with greater structural variability in our study with 52 molecular variants detected. However, most of them have a very low representation, and only 5-8 molecules have a relative abundance higher than 5% of all the molecules that make up each study group. Moreover, there is great variance in the intensity of each molecule in the different groups. Indeed, TG(52:2) is the most abundant molecule only in nevus melanocytes and metastatic melanoma as its intensity represents 13% and 17% respectively of all the TGs in each group. In skin melanocytes, although it represents 14.60%, similar to nevus and metastatic melanoma, TG(52:3) is the principal molecule of this group (15%). In addition to being the most abundant in skin melanocytes, the intensity of TG(52:3) is significantly lower in the other groups: 11% in nevus, 5% primary melanoma and 6% in metastatic melanoma. According to the most abundant specie in primary melanoma cells, this is TG(50:1), that increases its abundancy notably in primary cells comparing to skin (5%) and nevus (5%) melanocytes **(Appendix, Table 40)**.

On the other hand, only seven diglyceride variants have been detected in this study. Among these, DG(36:0) represents about 50% of the intensity of all the molecules identified in this class in the four study groups **(Appendix, Table 40)**.

Surprisingly, a global comparison of the relative abundance of triglycerides and diglycerides shows that there is a decreased presence in melanoma cell lines, compared to skin melanocytes **(Table 20, Fig. 34)**. Furthermore, skin melanocytes are very rich in triglycerides specially, since the intensity of this class is 3-fold higher compared to melanoma cell lines.

Table 20. Sum of the intensities obtained for all glycerolipid species (TG & DG) and comparison of the intensity variation of each class in the different study groups, related to control group (Melanocytes [M]=1). Legend: (M) Skin melanocytes: green; (N) nevus melanocytes: yellow; (MP) primary melanoma: red; (MM) metastatic melanoma: blue.

	Skin melanocytes (M)		Nevus melanocytes (N)		Primary Melanoma (MP)		Metastatic Melanoma (MM)	
	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M
TG	3,587,513	1.00	1,386,060	0.39	1,431,972	0.40	1,002,803	0.28
DG	162,028	1.00	119,738	0.74	96,811	0.60	128,269	0.79

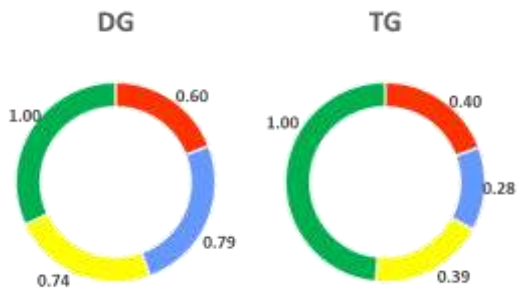


Figure 34. Comparison of the sum of the intensities obtained for diglycerides (DG) and triglycerides (TG) in the four study groups, related to skin melanocyte values (M=1). Legend: skin melanocytes (green), nevus melanocytes (yellow), primary (red) and metastatic (blue) melanoma.

1.2.3. Free fatty acids

This methodological approach enables the identification of free fatty acids as well. In this study, 24 FA molecular species were detected. Here, FA(18:0) is the principal molecule in the four study groups, making up 35% of the FFAs detected in skin melanocytes, 43% in nevus, 46% in primary melanoma and 47% in metastatic melanoma. However, only three of the FA molecules detected represent more than 5% of all the variants detected, thus, many of the lipids detected have a very low intensity although they can have an important role in the cells. In general, both melanoma groups present lower intensities of free fatty acids **(Table 21, Fig. 35)**.

Table 21. Sum of the intensities obtained for all lipid free fatty acid (FFA) species detected and comparison of the intensity variation of this class in the different study groups, related to control group (Melanocytes [M]=1). Legend: (M) Skin melanocytes: green; (N) nevus melanocytes: yellow; (MP) primary melanoma: red; (MM) metastatic melanoma: blue.

	Skin melanocytes (M)		Nevus melanocytes (N)		Primary Melanoma (MP)		Metastatic Melanoma (MM)	
	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M
FFA	5,413,034	1.00	5,349,715	0.99	4,015,087	0.74	3,345,719	0.62

Figure 35. Comparison of the intensities obtained for all the free fatty acid (FFA) species detected in the four study groups, related to skin melanocyte values (M=1). Legend: skin melanocytes (green), nevus melanocytes (yellow), primary (red) and metastatic (blue) melanoma.

1.2.4. Sphingolipids

In combination with the glycerophospholipids, sphingolipids are important components of the cell membranes. Using UHPLC-ESI-MS/MS, we have identified 8 different ceramide molecular species, 17 sphingomyelins and 6 hexosylceramides.

Table 22. Sum of the intensities obtained for all lipid the species that make up each lipid subclass within the sphingolipids and comparison of the intensity variation of each subclass in the different study groups, related to control group values (Melanocytes [M]=1). Legend: (M) skin melanocytes: green; (N) nevus melanocytes: yellow; (MP) primary melanoma: red; (MM) metastatic melanoma: blue.

	Skin Melanocytes (M)		Nevus Melanocytes (N)		Primary Melanoma (MP)		Metastatic Melanoma (MM)	
	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M	Intensity	Related to M
Cer	118,423	1.00	83,984	0.71	50,884	0.43	84,953	0.72
Short Cer	93,346	1.00	47,369	0.51	25,931	0.28	44,778	0.48
Long Cer	20,941	1.00	34,181	1.63	23,278	1.11	37,727	1.80
SM	783,788	1.00	651,501	0.83	169,613	0.22	245,063	0.31
HexCer	79,541	1.00	34,762	0.44	56,240	0.71	60,820	0.76

Regarding ceramides, there is a marked decrease in the intensity of the subclass in nevus (0.66) and melanoma cells (MP-0.47; MMGL-0.67), especially in primary melanoma **(Table 22, Fig. 36(A))**. Among these lipids, Cer(d18:1/16:0) is the principal molecule, as it comprises the 68% of the intensities detected for the ceramides in skin melanocytes, 56% in nevus melanocytes, 48% in primary melanoma and 49% in metastatic melanoma **(Appendix, Table 40)**. Notably, all the ceramides detected have a d(18:1) sphingoid base. However, each molecular variant has different fatty acid side-chains, which might determine the tendency of their presence in melanoma. Indeed, the ceramides with medium-chain fatty acids of 16 and 18 carbons, present very reduced levels in melanoma (MP-0.28; MMGL-0.48) and nevus (0.51) versus skin melanocytes (1.00), while long-chain (24 carbons) ceramides have increased presence in nevus (1.63) and metastatic melanoma (1.80) compared to melanocytes (1.00) **(Table 22, Fig. 36 (B))**.

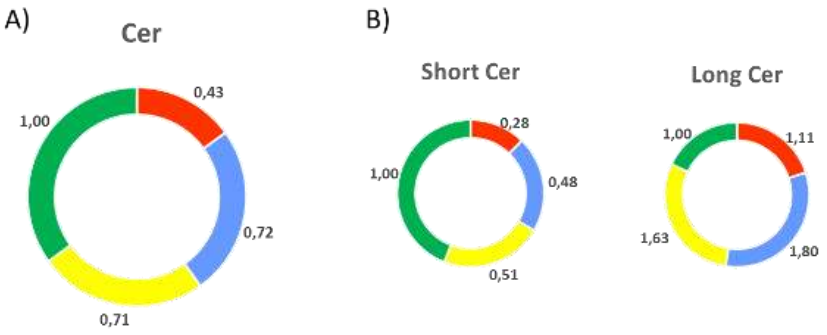


Figure 36. A) Comparison of the sum of the intensities obtained for all the detected ceramides (Cer) in the four study groups, related to skin melanocytes (M=1). B) Comparison of intensities of short and long-ceramides, related to skin melanocytes (M=1). Legend: skin melanocytes: green; nevus melanocytes: yellow: primary melanoma: red; metastatic melanoma: blue.

As well as ceramides, the relative abundance of sphingomyelins is intensily reduced from healthy melanocytes to malignant cells **(Table 22, Fig. 37)**. In this case, the principal molecule SM(d18:1/16:0) is shared among all the groups, representing 46-44% in melanocytic cells, and 28%-38% in primary and metastatic melanoma cells, respectively.

The last subclass of sphingolipids detected are the hexosylceramides, which are represented by six different molecular species. Like in the other sphingolipid subclasses, these lipids present a lower intensity especially in nevus melanocytes compared to skin melanocytes **(Table 22, Fig. 37)**.

Figure 37. Comparison of the sum of intensities obtained for all the sphingomyelin (SM) species and hexosylceramide (HexCer) species detected in the four study groups, related to skin melanocytes (M=1). Legend: skin melanocytes: green; nevus melanocytes: yellow; primary melanoma: red; metastatic melanoma: blue.

1.2.5. Cholesterol esters

The last lipid subclass detected is the cholesterol esters, which is represented by six different molecules. Here, the intensity decreases from skin melanocytes (1.00) to nevus (0.71) and melanoma cell lines (MP-0.79; MMGL-0.65) **(Appendix, Table 40; Fig. 38)**.

Figure 38. Comparison of the sum of intensities detected for all the cholesterol esters (CE) species in the four study groups, related to skin melanocytes (M=1). Legend: skin melanocytes: green; nevus melanocytes: yellow; primary melanoma: red; metastatic melanoma: blue.

1.3. Lipid biomarker discovery for malignancy

In the previous sections, we have demonstrated that the lipid profile of melanocytes and melanoma cells is different. Therefore, our next goal was to identify the individual lipid species that leaded the differentiation between the study groups. For this, orthogonal partial least squares discriminant analysis (OPLS-DA) was performed, as it identifies the molecules responsible for the maximum variation between groups.

Here, three different two to two comparisons were carried out for ESI+ and ESI- identified molecules. In **Figures 39 & 40**, non-malignant (M+N) and malignant cells (MP+MMGL) were compared. In the score-plots **(A)**, the more discriminant lipids are visualized, as these are the ones located on each edge of the plot. The blue colored lipids are more abundant in both melanocytes groups, while the red dots are the lipids with higher presence in malignant melanoma cells. In this comparison, 12 and 10 potential biomarkers for non-malignant cells have been identified in ESI+ and ESI- respectively, whereas five and six potential biomarkers for malignancy were detected in ESI+ and ESI- respectively. These results were confirmed in the VIP plots **(B)** and loading plots with jack-knifed confidence intervals **(C)**.

In total, in this study 33 possible biomarkers were identified in the comparison of the lipid profiles of non-malignant and malignant cells **(table 23)**, 17 in ESI+ and 16 in ESI-. Indeed, 22 are more abundant in melanocytes and 11 in malignant cells. Among the formers, there are six sphingomyelins, six triglycerides, six free fatty acids, one phosphatidylethanolamine, two ethanolamine ether lipids, and one phosphatidylglycerol. Conversely, the potential biomarkers that are more abundant in malignant cells are five choline ether lipids, two ethanolamine plasmalogens, two phosphatidylinositols, and two phosphatidilglycerol species.

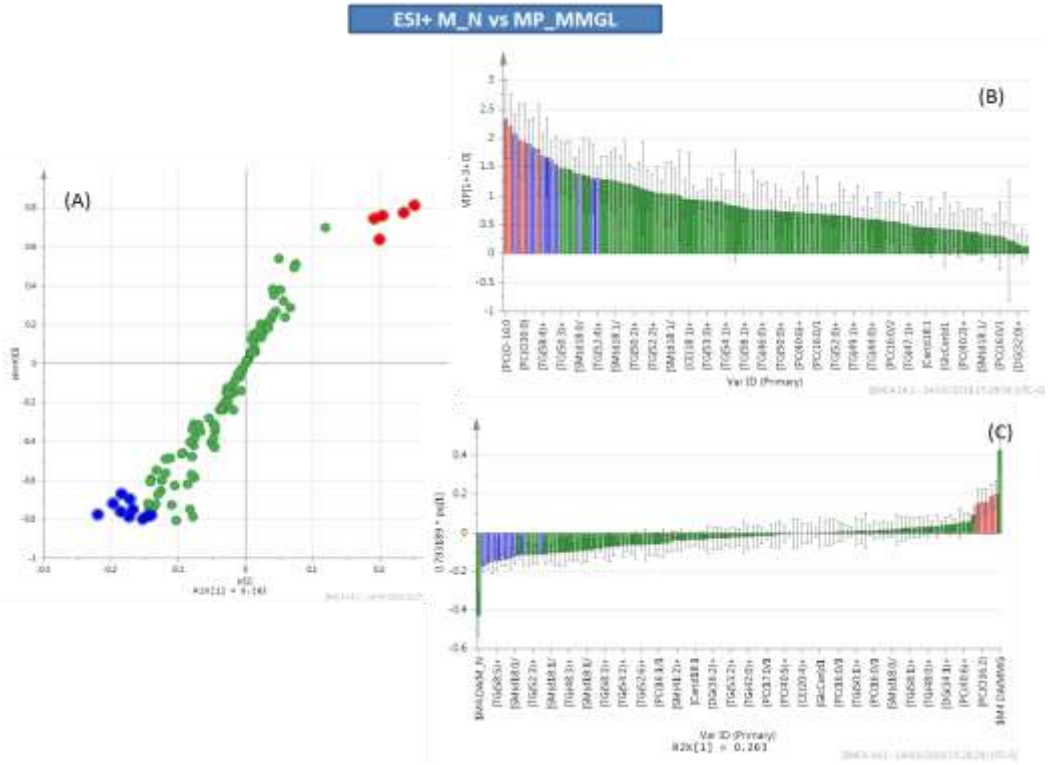


Figure 39. Identification of the possible lipid biomarkers for healthy and malignant cells in ESI+. A) Score plot of OPLS-DA shows that there are 12 potential markers for healthy cells (blue) and 5 for malignant cells (red). These results are confirmed in VIP plots (B) and loading plots with jack-knifed confidence intervals (C).

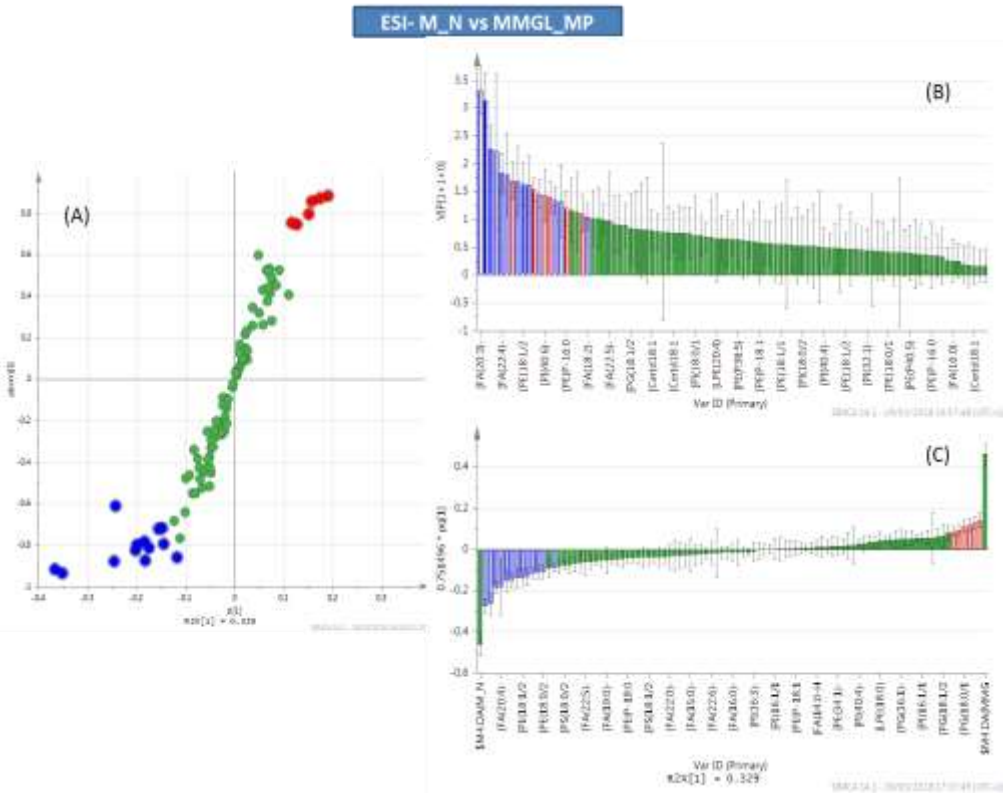


Figure 4039. Identification of the possible lipid biomarkers for healthy and malignant cells in ESI-. A) Score plot of OPLS-DA shows that there are 10 potential markers for healthy cells (blue) and 6 for malignant cells (red). These results are confirmed in VIP plots (B) and loading plots with jack-knifed confidence intervals (C).

Table 23. List of lipid potential biomarkers to discriminate between non-malignant (M+N) and malignant cells (MP+MMGL).

More abundant in (M+N)	More abundant in (MP+MMGL)
SM(d18:1/16:0)	PC(O30:0)
SM(d18:0/16:0)	PC(O32:1) and/or PC(P32:0)
SM(d18:1/22:0)	PC(O-16:0/16:0)
SM(d16:1/24:1)	PC(O-16:0/181)
SM(d18:1/24:1)	PC(O-36:2) and/or PC(P36:1)
SM(d18:1/26:1)	PE(P-16:0/20:5)
TG(50:4)	PE(P-16:0/22:6)
TG(52:4)	PI(40:6)
TG(56:5)	PI(40:5)
TG(56:4)	PG(32:0)
TG(58:5)	PG(18:0/16:1) and/or PG(18:1/16:0)
TG(58:4)	
FA(20:4)	
FA(20:3)	
FA(20:2)	
FA(22:4)	
FA(22:3)	
FA(22:2)	
PE(18:1/20:2) and/or PE(18:0/20:3)	
PE(P38:4) and/or PE(O38:5)	
PE(P38:3) and/or PE(O38:4)	
PG(18:1/20:2)	

In addition, we have also performed the comparison between individual groups. For instance, we studied the differences between nevus melanocytes and primary melanoma cells, since nevus can be sometimes the previous step to melanoma development, so detecting the differences between these cell lines can help in the identification of the molecules that can lead the malignant transformation of nevus melanocytes. As it can be observed in **Figures 41 & 42**, the score plots **(A)**, VIP plots **(B)** and loading plots **(C)** reveal that there are five potential markers that increase their intensity in primary melanoma cells in ESI+ and six in ESI-. On the other hand, there are eleven and ten lipid molecules in ESI + and ESI- respectively that decrease their intensity in this study group.

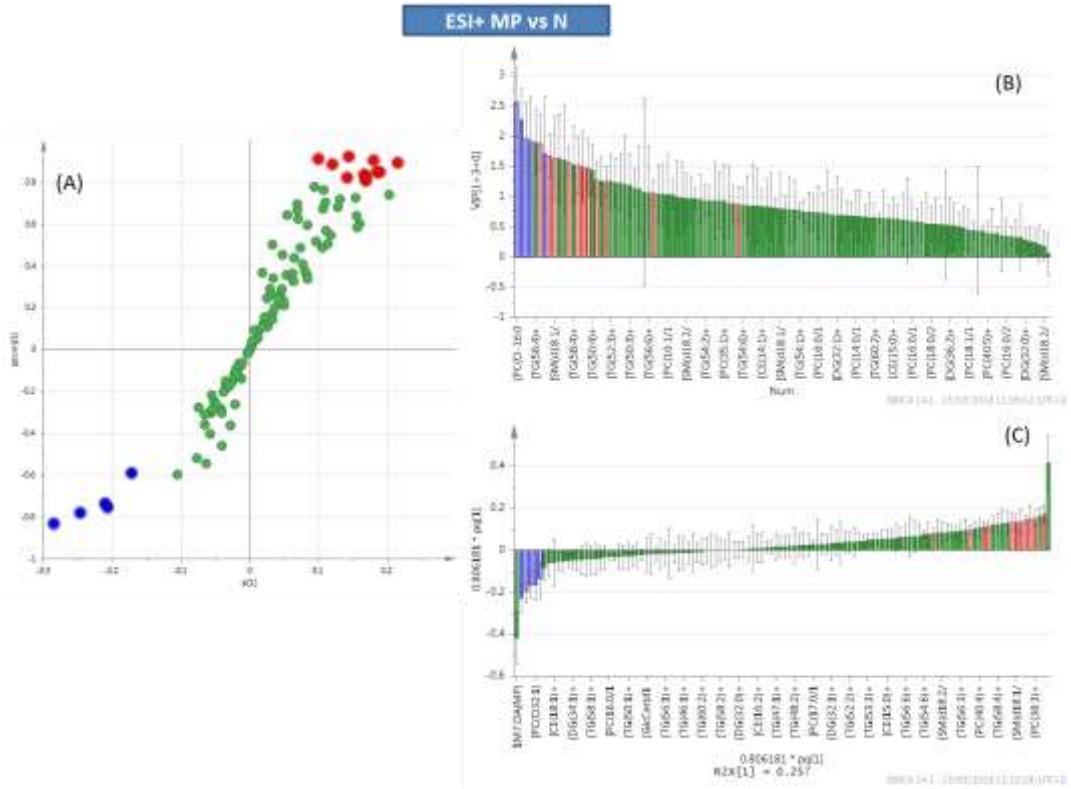


Figure 40. Identification of the potential lipid biomarkers for nevus melanocytes and primary melanoma cells in ESI+. A) Score plot of OPLS-DA shows that there are 5 potential markers for primary melanoma cells (blue) and 11 for nevus melanocytes (red). These results are confirmed in VIP plots (B) and loading plots with jack-knifed confidence intervals (C).

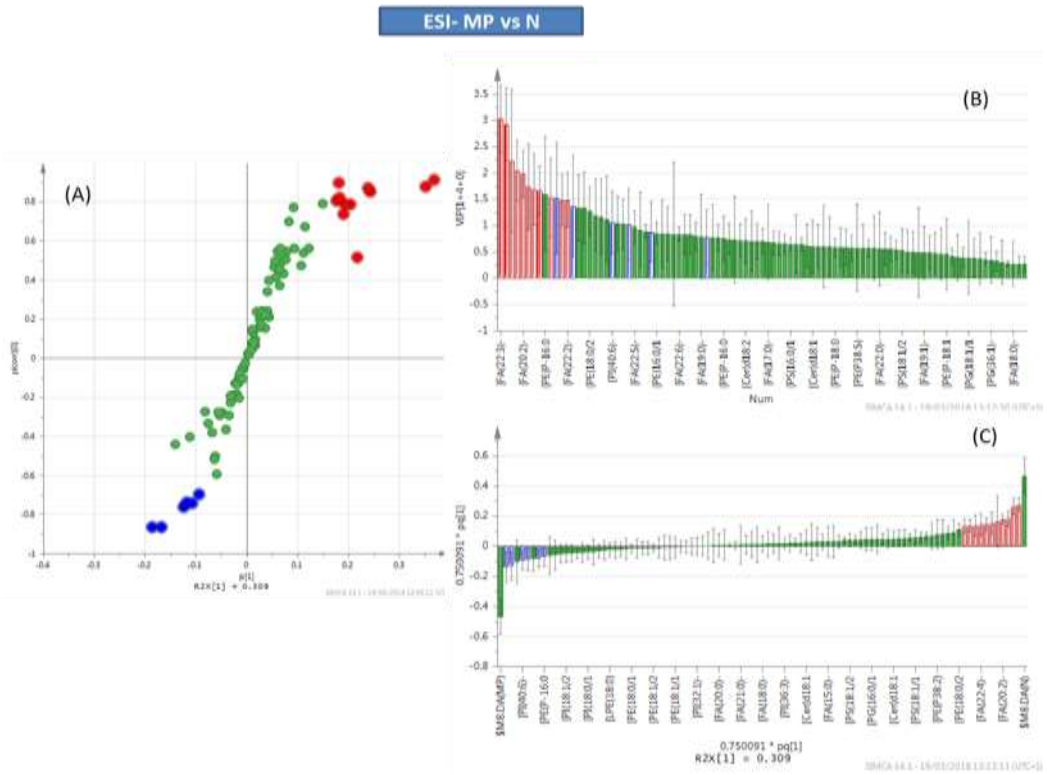


Figure 41. Identification of the potential lipid biomarkers for nevus melanocytes and primary melanoma cells in ESI-. A) Score plot of OPLS-DA shows that there are 6 potential markers for primary melanoma cells (blue) and 10 for nevus melanocytes (red). These results are confirmed in VIP plots (B) and loading plots with jack-knifed confidence intervals (C).

Thirty-two potential biomarkers were identified, which can make the difference between nevus melanocytes and malignant primary melanoma cells **(table 24)**. Twenty-one of these have higher presence in nevus melanocytes, while 11 increase their intensity in primary melanoma. Among the nevus markers, there are seven sphingomyelins, three triglycerides, six free fatty acids, one phosphatidylcholine, one phosphatidylethanolamine, two plasmalogens, and one phosphatidylglycerol. Conversely, five choline ether lipids, two phosphatidylethanolamine plasmalogens, two phosphatidylinositol and two phosphatidylglycerol increase their abundance in primary melanoma cells.

Table 24. List of lipid potential biomarkers to differentiate between nevus (N) and primary melanoma (MP) cells.

More abundant in N	More abundant in MP
SM(d18:1/16:0)	PC(O30:0)
SM(d18:0/16:0)	PC(O32:1) and/or PC(P32:0)
SM(d18:1/22:0)	PC(O-16:0/16:0)
SM(d16:1/24:1)	PC(O-16:0/181)
SM(d18:1/24:1)	PC(O-36:2) and/or PC(P36:1)
SM(d18:1/24:0) and/or SM(S18:0/24:1)	PE(P-16:0/20:5)
SM(d18:1/26:1)	PE(P-16:0/22:6)
TG(52:6)	PI(40:6)
TG(52:4)	PI(40:5)
TG(52:5)	PG(32:0)
FA(20:4)	PG(18:0/16:1) and/or PG(18:1/16:0)
FA(20:3)	
FA(20:2)	
FA(22:4)	
FA(22:3)	
FA(22:2)	
PC(38:3)	
PE(18:1/20:2) and/or PE(18:0/20:3)	
PE(P38:4) and/or PE(O38:5)	
PE(P38:3) and/or PE(O38:4)	
PG(18:1/20:2)	

The third comparison carried out in OPLS-DA was between nevus and metastatic melanoma cell lines. **Figures 43 & 44** show that there are 5 and 13 lipid species that present differential higher levels in metastatic melanoma in ESI + and ESI -, respectively. On the other hand, 10 and 8 lipid species are potential markers for nevus in ESI+ and ESI-, respectively.

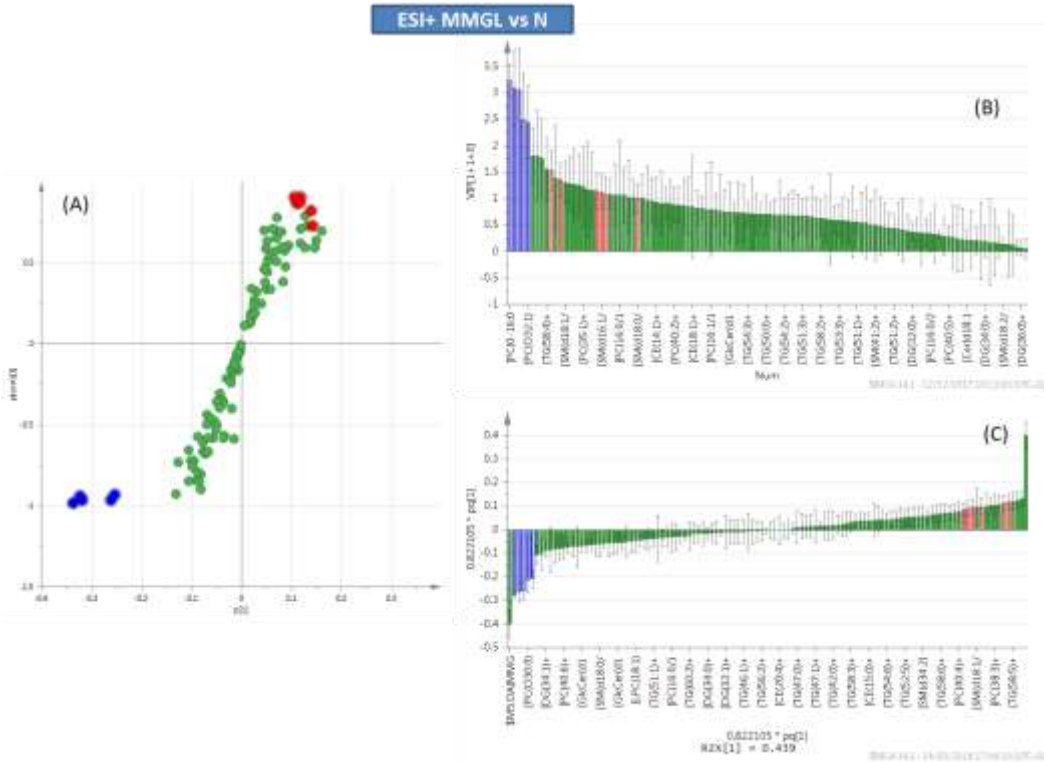


Figure 42. Identification of the potential lipid biomarkers for nevus melanocytes and metastatic melanoma cells in ESI+. A) Score plot of OPLS-DA shows that there are 5 potential markers for metastatic cells (blue) and 10 for nevus melanocytes (red). These results are confirmed in VIP plots (B) and loading plots with jack-knifed confidence intervals (C).

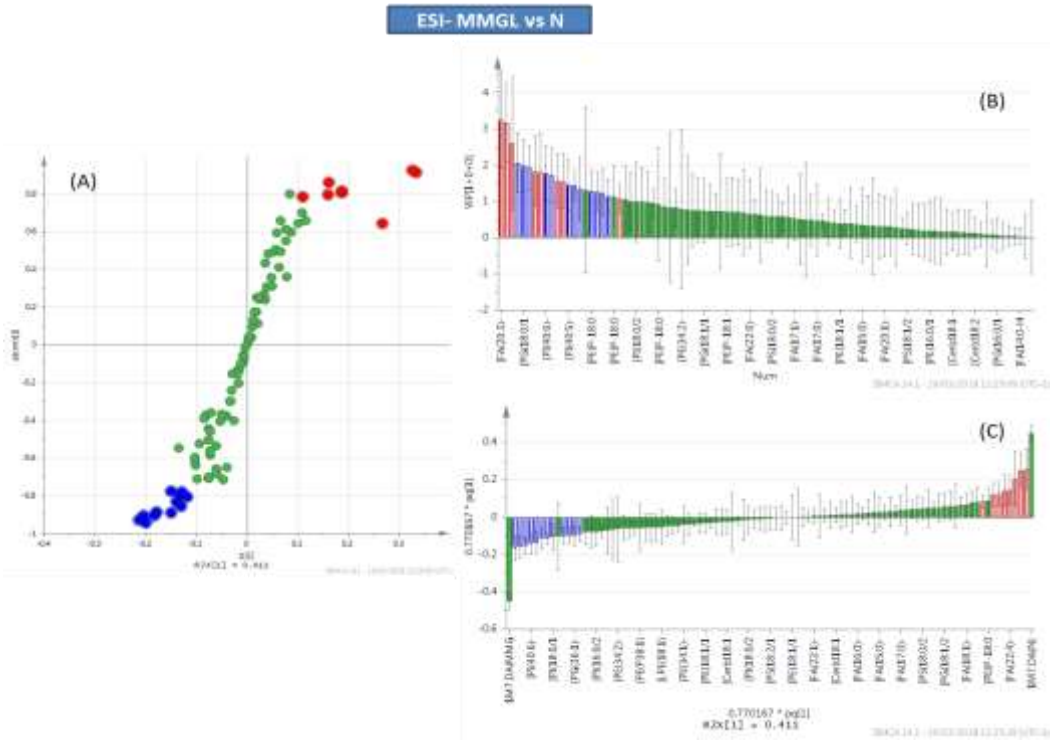


Figure 43. Identification of the potential lipid biomarkers for nevus melanocytes and metastatic melanoma cells in ESI-. A) Score plot of OPLS-DA shows that there are 13 potential markers for metastatic cells (blue) and 8 for nevus melanocytes (red). These results are confirmed in VIP plots (B) and loading plots with jack-knifed confidence intervals (C).

As it was expected, the comparison between nevus melanocytes and metastatic melanoma cells lead to the identification of the greater number of possible biomarkers. Indeed, there are 36 lipid species **(table 25),** that present an intensity different enough to discriminate between these two groups of cells. The potential biomarkers that are more abundant in nevus are five sphingomyelins, five triglycerides, five free fatty acids, one phosphatidylethanolamine and two plasmalogens. In contrast, there are another 18 lipids that show increased presence in metastatic melanomas: five choline ether lipids, three plasmalogens, seven phosphatidylinositols and three phosphatidylglycerols.

Table 25. List of lipid species that discriminate nevus melanocytes (N) from metastatic melanoma (MMGL) cells.

More abundant in N	More abundant in MMGL
SM(d18:1/14:0)	PC(O30:0)
SM(d18:1/16:0)	PC(O32:1) and/or PC(P32:0)
SM(d18:0/16:0)	PC(O-16:0/16:0)
SM(d16:1/24:1)	PC(O-16:0/181)
SM(d18:1/24:1)	PC(O-36:2) and/or PC(P36:1)
TG(50:4)	PE(P-16:0/20:5)
TG(56:5)	PE(P-16:0/22:6)
TG(56:4)	PE(P-18:0/22:6)
TG(58:5)	PI(34:1)
TG(58:4)	PI(18:0/18:2) and/or PI(18:2/18:0)
FA(20:4)	PI(14:0/22:1) and/or PI(16:0/20:1)
FA(20:3)	PI(18:1/20:4)
FA(20:2)	PI(18:0/20:2)
FA(22:4)	PI(40:6)
FA(22:3)	PI(40:5)
PE(18:1/20:2) and/or PE(18:0/20:3)	PG(32:0)
PE(P38:4) and/or PE(O38:5)	PG(18:0/16:1) and/or PG(18:1/16:0)
PE(P38:3) and/or PE(O38:4)	PG(36:1)

Finally, primary and metastatic melanoma lipid profiles were compared, in order to identify the lipids that drive the metastatic behavior of the cells. Here, six possible biomarkers were detected, all of which were detected in ESI+ and increased their presence accordingly to melanoma progression (**Fig. 45)**. Among these, two plasmalogens, one phosphatidylinositol and three phosphatidylglycerols were identified **(table 26)**.

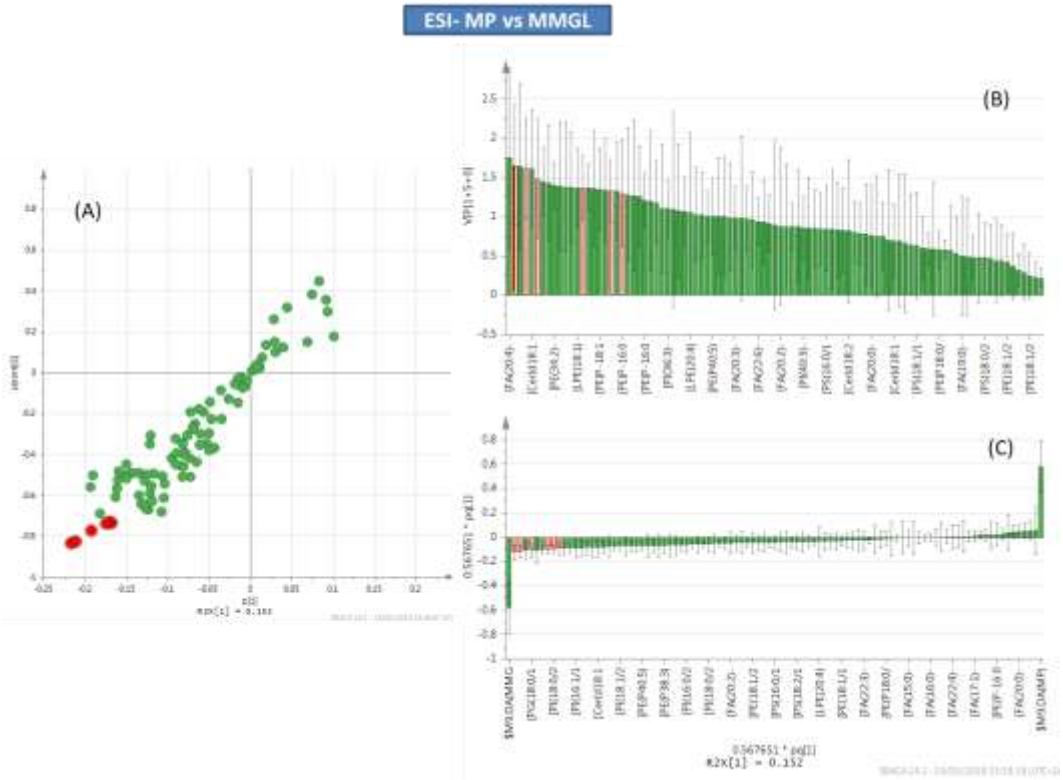


Figure 44. Identification of the potential lipid biomarkers for primary and metastatic melanoma cells in ESI-. A) Score plot of OPLS-DA shows that there are 6 potential markers for metastatic melanoma (red). These results are confirmed in VIP plots (B) and loading plots with jack-knifed confidence intervals (C).

Table 26. List of lipid potential biomarkers of metastatic melanoma (MMGL) compared to primary melanoma cells.

More abundant in MMGL
PE(P-16:0/22:6)
PE(P-18:0/22:6)
PI(18:0/20:2)
PG(18:0/16:1) and/or PG(18:1/16:0)
PG(36:1)
PG(18:1/20:2)

In summary, after applying OPLS-DA a panel of 45 lipid species that are able to discriminate the study groups has been identified **(table 27)**.

Table 27. Summary of the potential biomarkers. The lipid species in light grey are more abundant in non-pathological groups, whereas bold marks are for the lipid species that are more abundant in pathological cell lines.

Lipid potential markers	N+M vs **MP+MMGL**	N vs **MP**	N vs **MMGL**	MP vs **MMGL**
SM(d18:1/14:0)			X	
SM(d18:1/16:0)	X	X	X	
SM(d18:0/16:0)	X	X	X	
SM(d18:1/22:0)	X	X		
SM(d16:1/24:1)	X	X	X	
SM(d18:1/24:1)	X	X	X	
SM(d18:1/24:0) and/or SM(d18:0/24:1)		X		
SM(d18:1/26:1)	X	X		
TG(50:4)	X		X	
TG(52:6)		X		
TG(52:4)	X	X		
TG(52:5)		X		
TG(56:5)	X		X	
TG(56:4)	X		X	
TG(58:5)	X		X	
TG(58:4)	X		X	
FA(20:4)	X	X	X	
FA(20:3)	X	X	X	
FA(20:2)	X	X	X	
FA(22:4)	X	X	X	
FA(22:3)	X	X	X	
FA(22:2)	X	X		
PC(38:3)		X		
PC(O30:0)	**X**	**X**	**X**	
PC(O32:1) and/or PC(P32:0)	**X**	**X**	**X**	
PC(O16:0/16:0)	**X**	**X**	**X**	
PC(O16:0/18:1)	**X**	**X**	**X**	
PC(O36:2) and/or PC(P36:1)	**X**	**X**	**X**	
PE(18:1/20:2) and/or PE(18:0/20:3)	X	X	X	
PE(P38:4) and/or PE(O38:5)	X	X	X	
PE(P38:3) and/or PE(O38:4)	X	X	X	
PE(P16:0/20:5)	**X**	**X**	**X**	
PE(P16:0/22:6)	**X**	**X**	**X**	**X**
PE(P18:0/22:6)			**X**	**X**
PI(34:1)			**X**	
PI(18:0/18:2) and/or PI(18:2/18:0)			**X**	
PI(14:0/22:1) and/or PI(16:0/20:1)			**X**	
PI(18:1/20:4)			**X**	
PI(18:0/20:2)			**X**	**X**
PI(40:6)	**X**	**X**	**X**	
PI(40:5)	**X**	**X**	**X**	
PG(32:0)	**X**	**X**	**X**	
PG(18:0/16:1) and/or PG(18:1/16:0)	**X**	**X**	**X**	**X**
PG(36:1)			**X**	**X**
PG(18:1/20:2)	X	X		**X**

In order to visualize the differences in the relative abundance of each discriminant lipid in the four study groups, they were represented in Box-Whisker graphs. All the sphingomyelins, which are represented in **figure 46**, show the same trend. This is, their intensity is reduced with the development and progression of melanoma. Eight out of the seventeen sphingomyelins identified are potential biomarkers for this malignancy, giving insight into the importance of this lipid subclass in melanoma. The contribution of the discriminant lipids to the total intensity detected for SMs in each study group is not an important feature, as among the potential biomarkers there is SM(d18:1/16:0) whose intensity represents 46-28% of all the SMs, while SM(d18:1/26:1) has a scarce presence (0.34%-0.55%)

Triglycerides are also very represented as they have also eight discriminant lipid species. These molecules follow the same tendency as SMs, as their expression is markedly higher in melanocytes **(fig. 47)**. Notably, all the potential biomarkers of this subclass have a very low presence in the group, with relative percentages ranging from 0.09% to 4.40%, suggesting that even lipids with very low presence in the cells can perform critical roles in tumor development.

As it can be observed in **figure 48,** similar trends and results were achieved for free fatty acids. Here, six FFAs have the ability to discriminate between non-malignant and malignant cells, clearly reducing their intensity in both melanoma groups. Furthermore, lipids with little presence are able to discriminate the study groups. For example, the molecule FA(22:3) represents the 0,37-0,34% of the intensities detected for all the FFAs in non-malignant cells, and decreases its intensity 18-fold in melanoma cells.

Phosphatidylinositols on their side are represented by seven potential biomarkers, showing all of them an increased intensity in melanoma, especially in metastatic cells **(fig. 49)**.

According to the ethanolamine lipids, one PE and five PE ether lipids have been detected as potential biomarkers **(fig. 50)**. However, there is not a general tendency within the group, since the PE and two of the ether lipids show decreased intensity in melanoma, whereas the three ether lipids increase their presence in the malignancy, in the metastatic stage specially.

Despite the general increase observed for choline bearing glycerophospholipids in melanoma, the potential biomarker PC(38:3) presents decreased levels in both melanoma groups **(fig. 51)**. Interestingly, the five choline ethers that have been identified by this methodological approach have been described as potential biomarkers in our study. Furthermore, every one of them increase their levels accordingly to melanoma progression.

It must be pointed out that ten out of the 45-discriminant lipids are ether lipids, therefore highlighting the importance that peroxisomes might have in melanoma development and progression.

The last subclass represented among the potential biomarkers are the phosphatidylglycerols **(fig. 52)**. Here, half of the PGs detected show discriminant levels. Among these, PG(32:0) increases its intensity in the malignancy. Furthermore, two other PGs, PG(18:0/16:1) and/or PG(18:1/16:0) and PG(36:1), augment their levels in melanoma, which increase accordingly to the progression of the malignancy, being able to discriminate between primary and metastatic melanoma. Markedly, PG(18:0/16:1) and/or PG(18:1/16:0) goes from being the fifth more abundant PG in skin melanocytes, to be the first and second most abundant in metastatic and primary melanoma, respectively.

As it can be observed in these Box-Whisker representations **(fig. 46-52)** of all the potential biomarkers detected, the boxes are overlapped with the ones of other study groups. Therefore, there is no single lipid on its own that can differentiate between the groups. Nonetheless, we have documented a panel of 45 lipids that all together discriminate between healthy and malignant cells, and the progression stage of the melanoma.

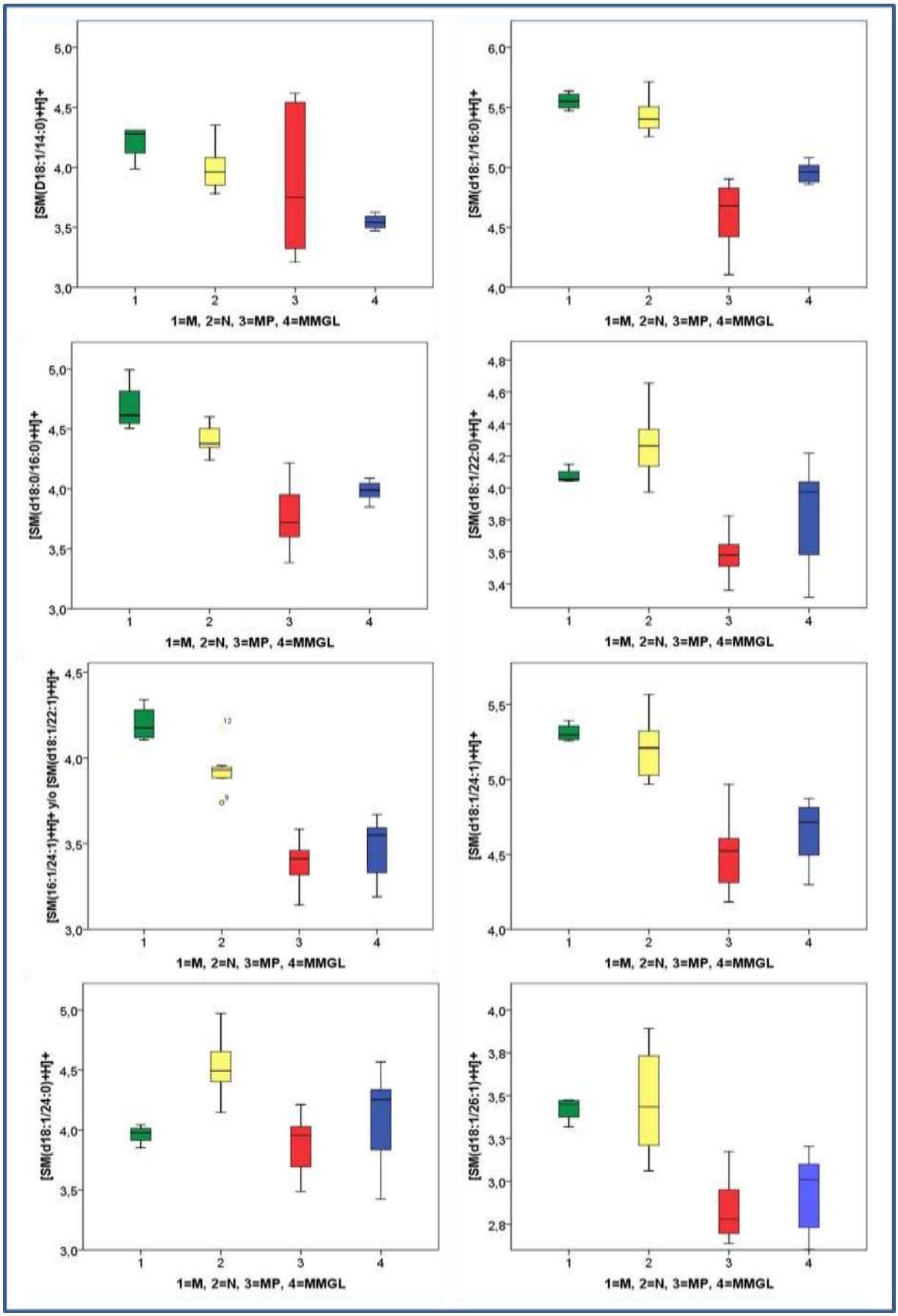


Figure 45. Box-Whisker graphs for the SMs identified as potential biomarkers for melanoma detected in skin melanocytes (column 1- green), nevus melanocytes (column 2- yellow), primary melanoma (column 3- red) and metastatic melanoma (column 4 - blue).

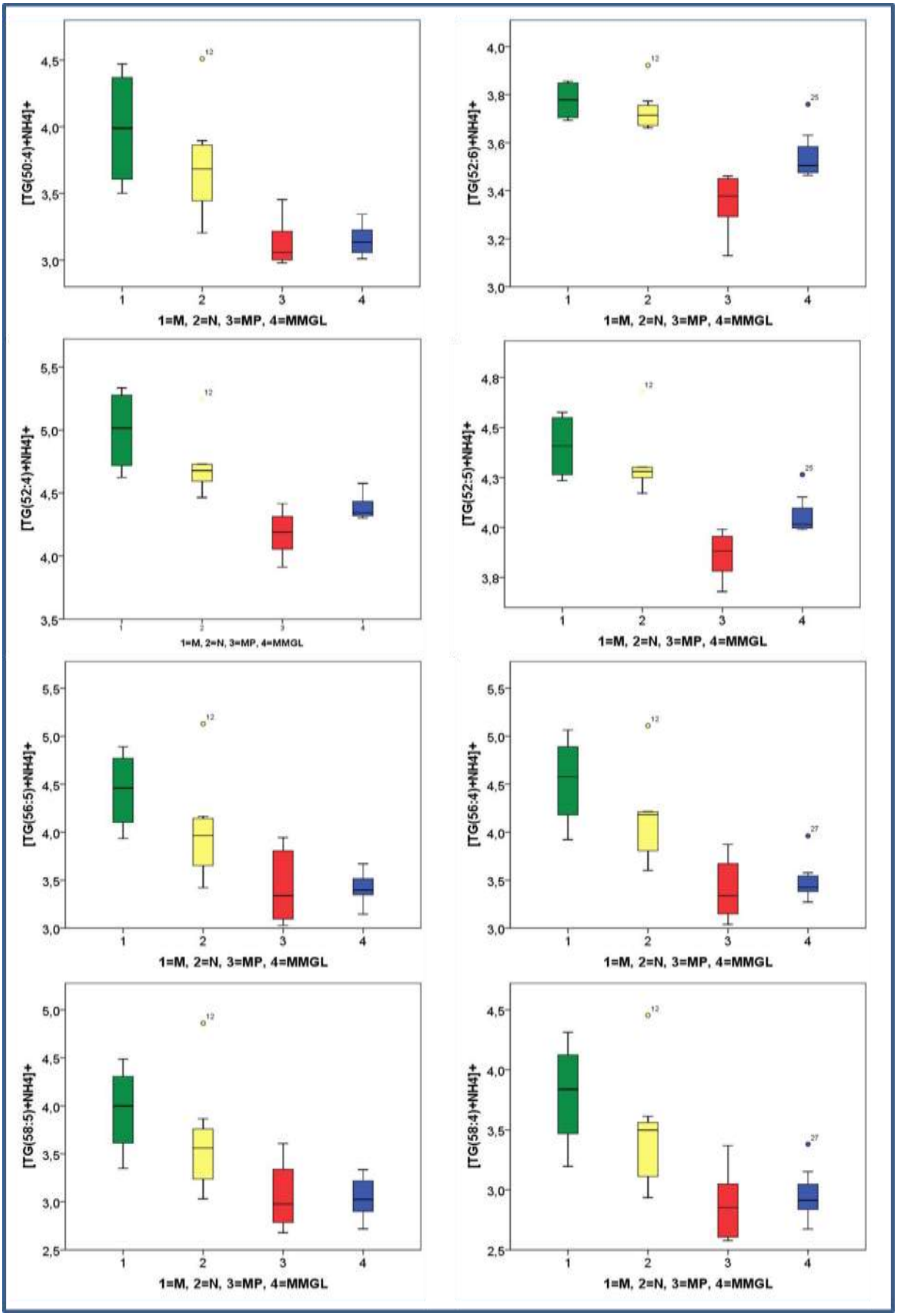


Figure 46. Box-Whisker graphs for the TGs identified as potential biomarkers for melanoma detected in skin melanocytes (column 1- green), nevus melanocytes (column 2- yellow), primary melanoma (column 3- red) and metastatic melanoma (column 4 - blue).

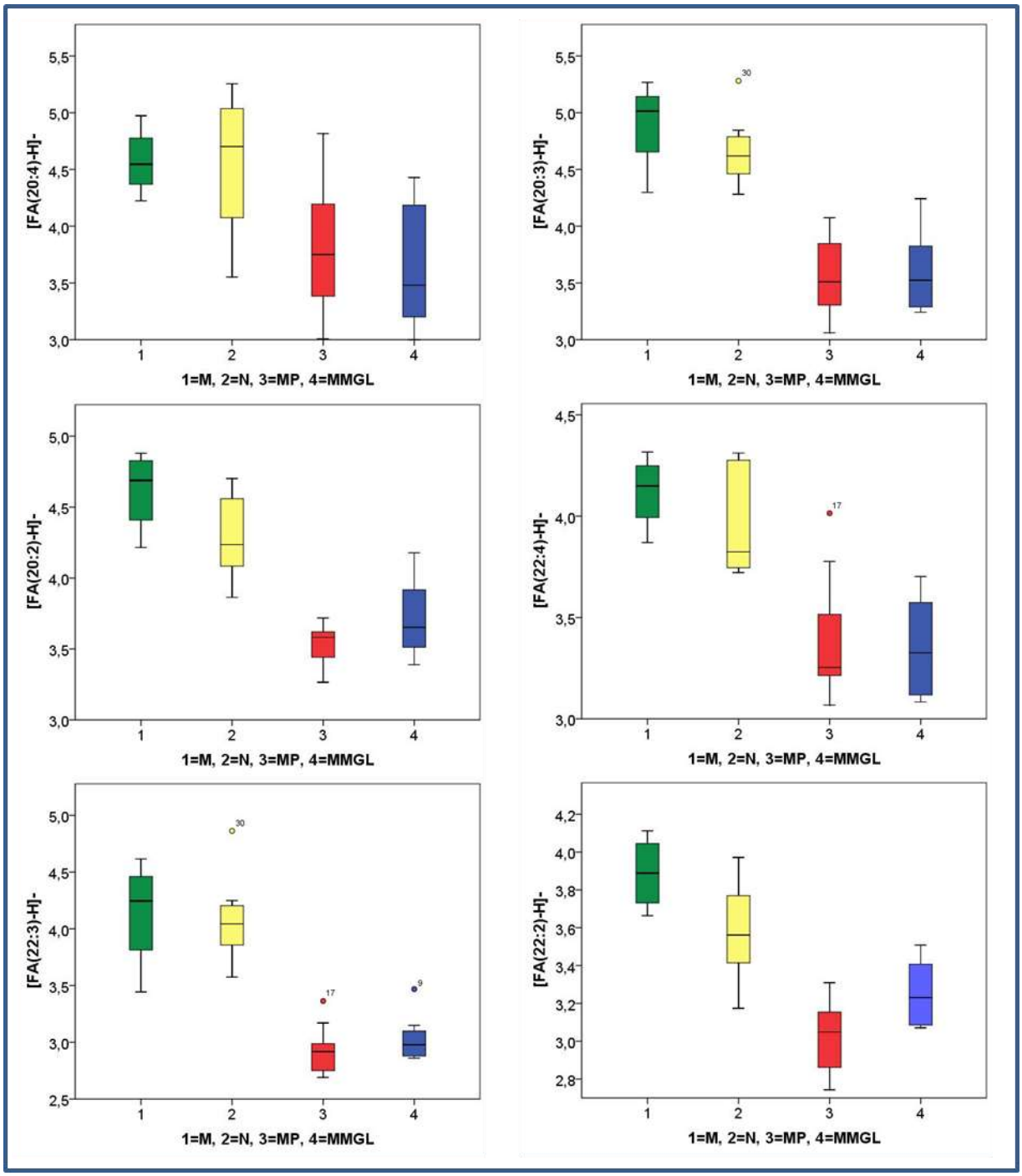


Figure 47. Box-Whisker graphs for the FAs identified as potential biomarkers for melanoma detected in skin melanocytes (column 1- green), nevus melanocytes (column 2- yellow), primary melanoma (column 3- red) and metastatic melanoma (column 4 - blue).

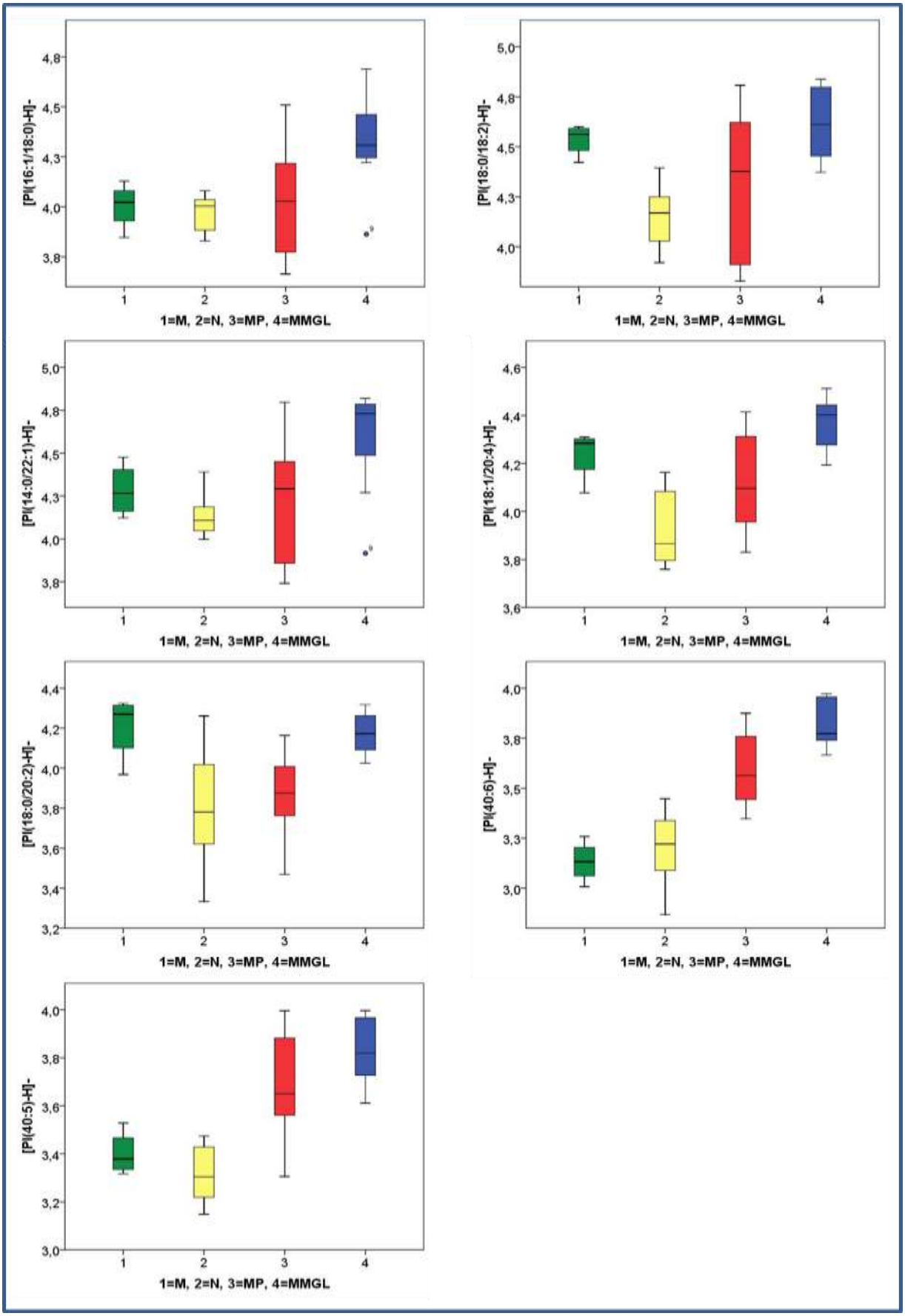


Figure 48. Box-Whisker graphs for the PIs identified as potential biomarkers for melanoma detected in skin melanocytes (column 1- green), nevus melanocytes (column 2- yellow), primary melanoma (column 3- red) and metastatic melanoma (column 4 – blue).

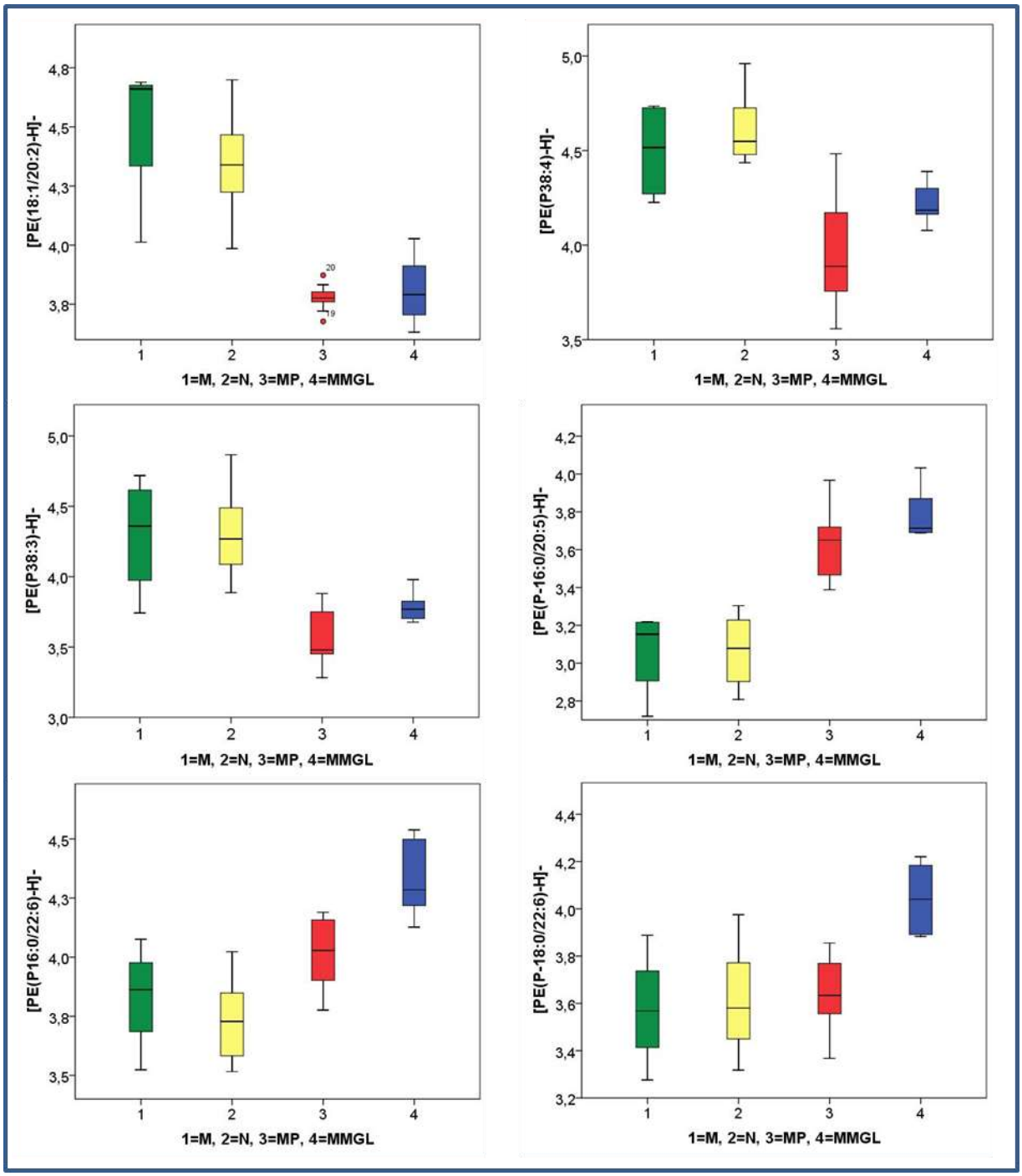


Figure 49. Box-Whisker graphs for the ethanolamine lipids identified as potential biomarkers for melanoma detected in skin melanocytes (column 1- green), nevus melanocytes (column 2- yellow), primary melanoma (column 3- red) and metastatic melanoma (column 4 - blue).

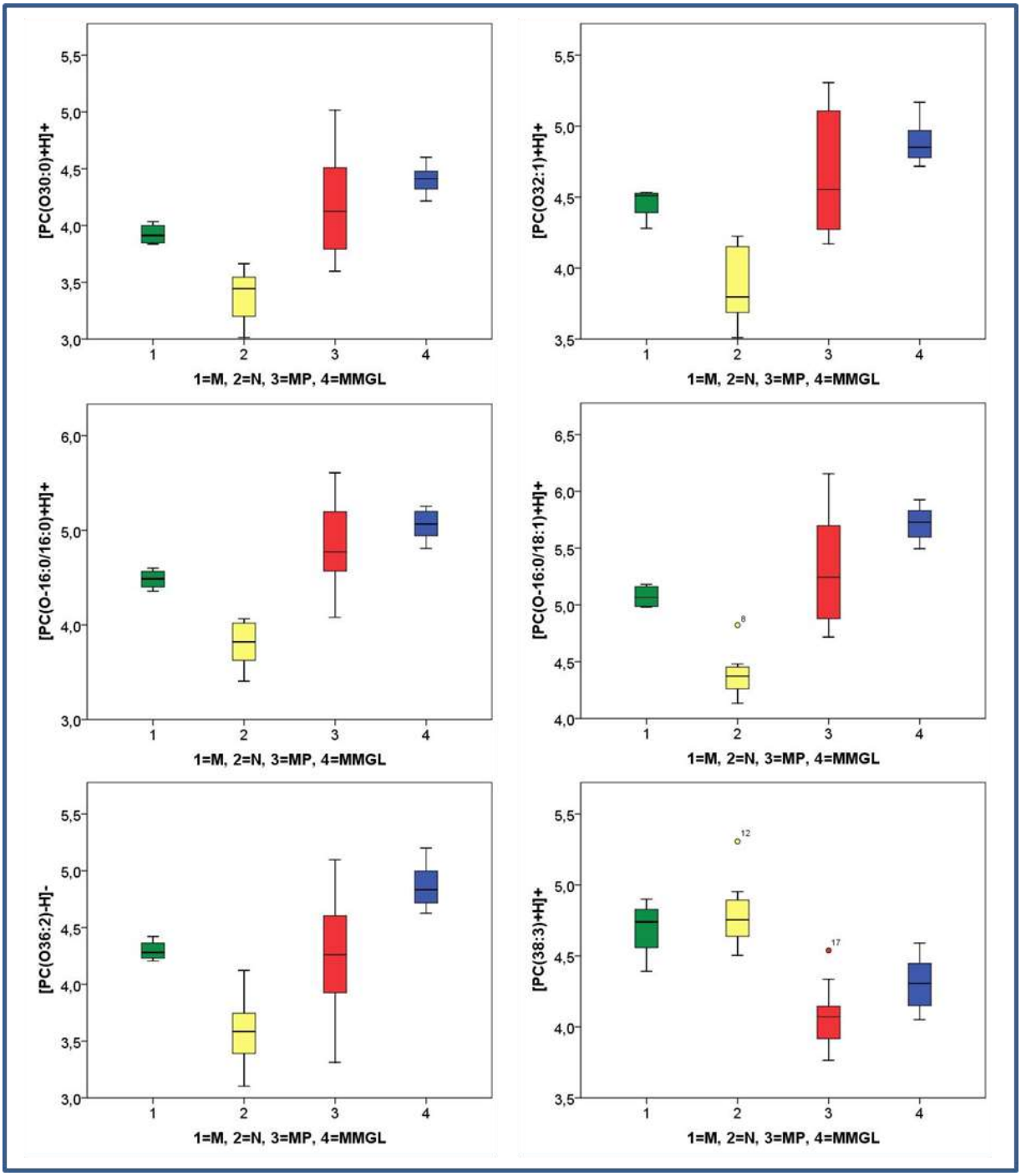


Figure 50. Box-Whisker graphs for the choline lipids identified as potential biomarkers for melanoma detected in skin melanocytes (column 1- green), nevus melanocytes (column 2- yellow), primary melanoma (column 3- red) and metastatic melanoma (column 4 - blue).

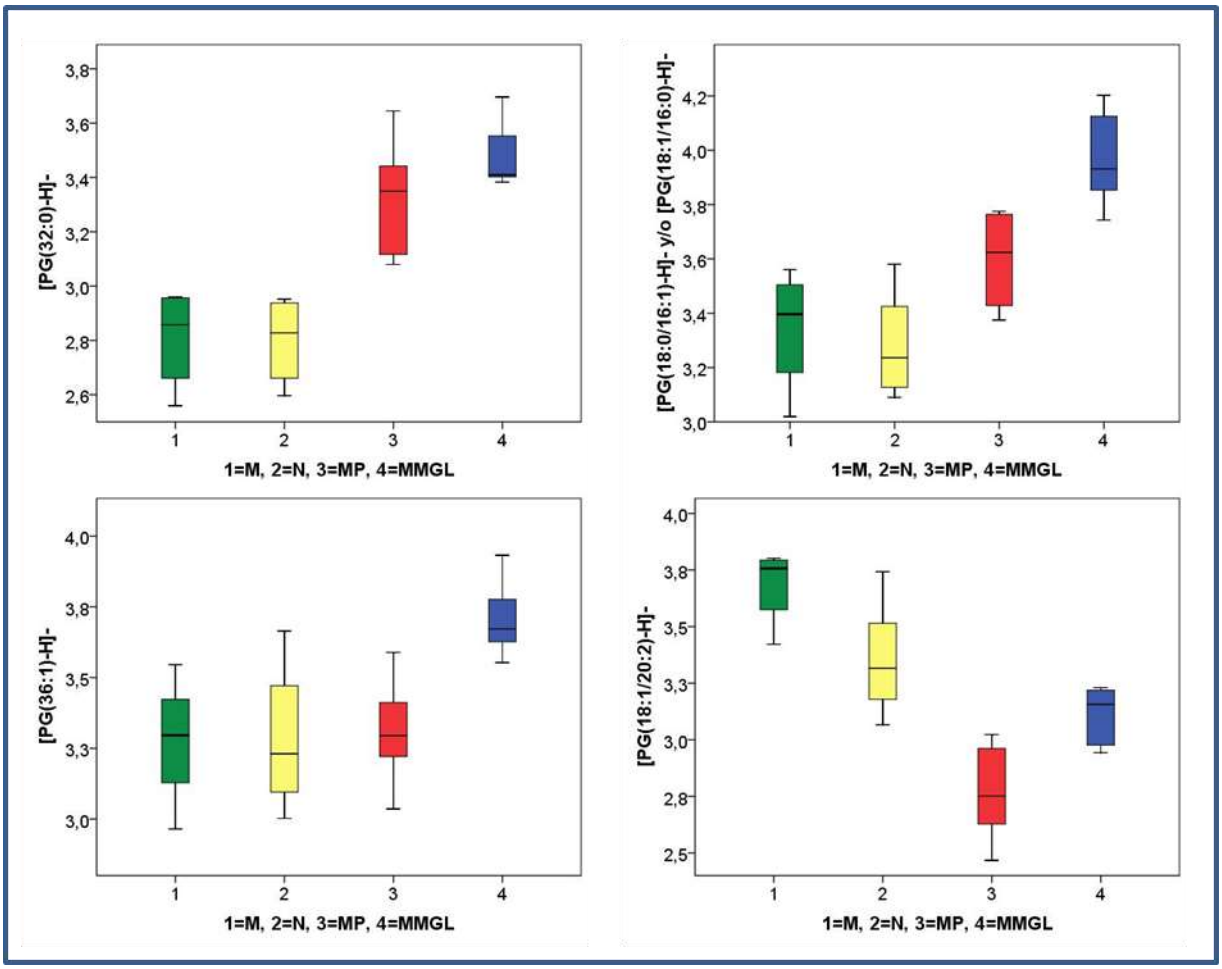


Figure 51. Box-Whisker graphs for the PGs identified as potential biomarkers for melanoma detected in skin melanocytes (column 1- green), nevus melanocytes (column 2- yellow), primary melanoma (column 3- red) and metastatic melanoma (column 4 - blue).

2. Lipidomic analysis of functional membrane microarrays

The results obtained in this section are encoded as they are object of patenting in the patent registered as follows: Oncofinder: A novel platform for screening benignant nevi from melanomas based on lipid phenotype using mass spectrometry and machine learning.

The results of the previous section validated our hypothesis, which claimed that the lipidome of healthy melanocytes and melanoma cells is different. As described in the materials and methods section (2.3.4.), the UHPLC-ESI-MS/MS technology employed has some limitations, including the need of numerous technical steps and limitations for translation into clinics; therefore, the next objective was to detect new biomarkers for melanoma by studying a broader number of cell lines and replicates using a biotechnological tool with translational potential. For this, we studied the lipid composition of cell membranes present in the microarrays fabricated by IMG Pharma company using an own methodology. Here, the cell membrane suspensions of three normal skin melanocyte cell lines (HEMn-LP, HEMn-MP, and HEMn-DP), nine cell lines of melanocytes isolated from nevi (N1, N2, N3, N4, N5, N6, N7, N8, N9), six commercial primary melanomas (A375, Mel-Ho, Sk-Mel-28, Sk-Mel-31, G-361, ME4405) and nine metastatic melanoma cell lines (Colo-800, A2058, Hs294T, HT144, Sk-Mel-3, Sk-Mel-2, RMPI 7951, VMM1) were placed. Three replicates of different passages of each cell line were printed.

The previous results revealed that most of the significant lipids detected are found in cell membranes, thus, this could be an appropriate approach to precisely study the lipidome of the samples and detect differently expressed lipids with no need of lipid extraction and employing very little quantity of each sample.

For the lipidomic analysis, the group of Dr. Fernández in the UPV/EHU analyzed the lipid content of each spot of the microarray performing a MALDI mass spectrometry protocol. In order to determine the reproducibility of the methodological approach, five different arrays were analyzed in different days for both positive and negative ionization modes, using different matrixes: MBT for positive ionization and DAN for negative ionization.

Once all the mass spectra were collected, the first step was to identify all the mass channels that corresponded to lipid molecules. Then, the data obtained were classified using a divisive hierarchical clustering in order to visualize the natural aggrupation of the samples. A color scale was used for this, and the spots were colored based on 1-correlation between clusters, so the more similar the clusters are the closer their color is in the color scale. The results indicate that the three skin melanocyte samples that were placed in different locations of the array are classified together, thus, there is no intensity fluctuation during the experiment. Taken together the five arrays examined, the formed clusters are consistent over the experiments carried out in different days (**Fig. 53 B)**. Therefore, these results suggest that there is good-intra and -inter experimental reproducibility.

Moreover, this classification model illustrates the differences between the lipid content of benign and tumor cells. Indeed, when the clustering level is set to five clusters, all the melanocytes cell lines are grouped together (white spots in **Fig. 53 B**), while melanoma cells are distributed in the remaining four groups. Furthermore, if the level of clustering is increased to 15 Cl, skin (white and pink spots) and nevus (purple spots) melanocytes separate, demonstrating that they have a different lipid phenotype. However, their color is closer in the color scale than the color of melanoma cell lines, which suggests that although their lipid content is different, it is more similar than to melanoma. However, this model could not discriminate between primary and metastatic melanoma cells.

A)

		HEM-MP p6	SKMEL-3 p6	WM-266 p4	SKMEL31 P4	Nevus 1 p6	Nevus 4 p4	Nevus 7 p4	HEM-MP p6
		HEM-DP p8	SKMEL-3 p7	WM-266 p5	SKMEL31 P6	Nevus 2 p5	Nevus 5 p3	Nevus 8 p3	HEM-DP p8
		HEM-LP p9	SKMEL-3 p8	WM-266 p6	SKMEL31 P8	Nevus 3 p4	Nevus 6 p8	Nevus 9 p4	HEM-LP p9
A375 p14	Hs294T p5	RPMI p19	A2058 p5	SKMEL28 p3	ME4405 p4	HEM-MP p6	HEM-MP p6	VMM1 p12	SKMEL-2 p5
A375 p15	Hs294T p6	RPMI p20	A2058 p6	SKMEL28 p5	ME4405 p5	HEM-MP p5	HEM-MP p5	VMM1 p14	SKMEL-2 p6
A375 p16	Hs294T p7	RPMI p21	A2058 p7	SKMEL28 p7	ME4405 p6	HEM-MP p9	HEM-MP p9	VMM1 p15	SKMEL-2 p8
						MEL-Ho p19	G361 p6	COLO800 p25	HT144 p15
						MEL-Ho p20	G361 p7	COLO800 p26	HT144 p16
						MEL-Ho p21	G361 p8	COLO800 p27	HT144 p17

B)

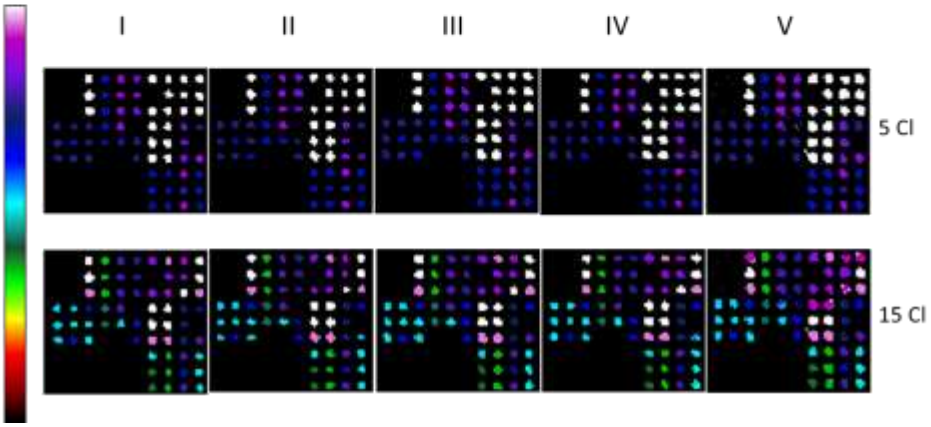


Figure 52. A) Schema of the sample distribution in the cell membrane microarrays. Legend: skin melanocytes: green; melanocytes isolated from nevus: yellow; primary melanomas: red; metastatic melanomas: blue. **B) Clustering aggrupation of the samples in 5 clusters (Cl) and 15 clusters (Cl), representing the similarity based on the color scale.**

Although some lipid species present different adducts, we considered each of them individual molecules. This is, we included in the analysis different adducts of the same lipid molecule. In total, 271 mass channels that corresponded to lipid molecules were detected **(Fig. 54)**, spanning 24 sphingomyelin molecules (SM), 1 hexosylceramide (HexCer), 3 diacylglycerides (DG), 11 triacylglycerides (TG), 64 phosphatidylcholines (PC), 31 choline ether lipids (PC(P/O)), 23 phosphatidylethanolamines (PE), 17 ethanolamine ether lipids (PE(P/O)), 24 molecules that could be either PC or PE, 15 PC or PE ether lipids, 11 phosphatidylserines (PS), 16 phosphatidylglycerols (PG), 1 glycerol ether lipid (PG(P/O)), 28 phosphatidylinositols (PI) and 2 inositol ether lipids (PI(P/O)).

MALDI mass spectrometry is an analytical technique with soft ionization; therefore, there is minimal fragmentation of the ions created, which impedes the identification of the side-chains of the lipid molecules. In order to overcome this disadvantage, in this work, the structural information achieved in the UHPLC-ESI-MS/MS approach has been used for the lipid species that were identified in both MS methodologies. In this way, the acyl/alkyl/alkenyl side-chains were annotated to the lipid species identified in MALDI providing a better description of the molecule.

However, some of the molecules were not detected in the ESI methodology, and it has not been possible to determine their acyl side-chain description or if they are PC or PE, for example.

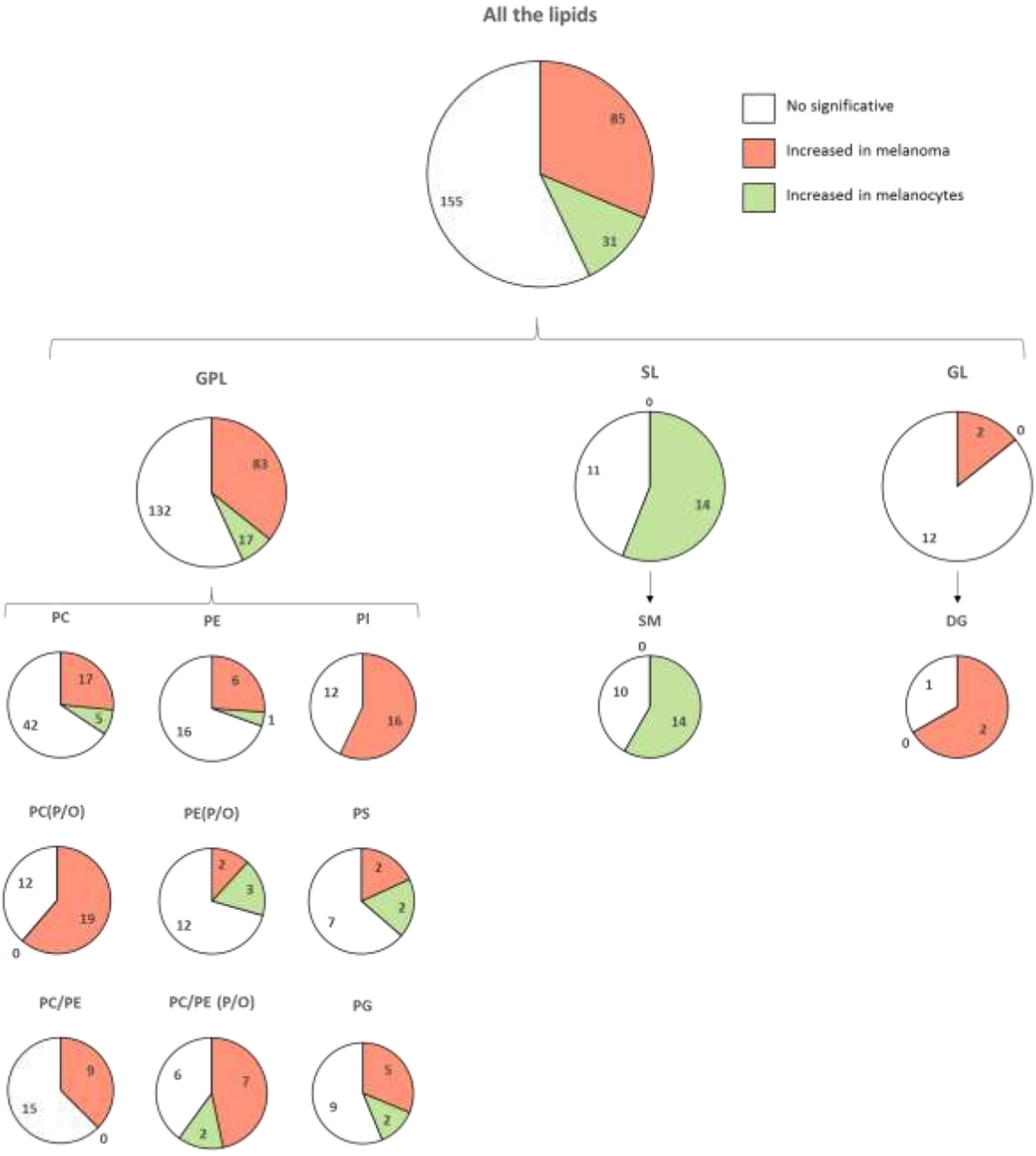


Figure 53. Overview of the lipid species detected in MALDI-MS and representation of their significance to differentiate melanocytes from melanoma cells. The number within each section of the pie chart depicts the number of species that represent the section.

A general t-test ($p<0.05$) confronting non-malignant and malignant cell lines was conducted to study the potential biomarkers that are differentially expressed in these two groups, and therefore confirm that the methodology applied is useful to detect the lipid species that are capable of distinguishing normal and tumor cells. Here, 114 lipid species showed significantly different intensities, where 29 presented enhanced levels in non-malignant cell lines, whereas 85 increased their expression with the development of melanoma **(Table 28)**. Indeed, melanocytes were represented by increased levels in 14 SM molecules, whereas tumor cell lines were characterized by presenting enhanced intensity in 2 DGs, 19 PC ether lipids, 9 PC or PE molecules, and 16 PI species. In addition, among the potential biomarkers, 22 significant PC molecules were identified, 5 of these showed an increased presence in melanocytes, while 17 represented melanoma tissue. Ethanolamine phospholipids on their side contributed with 7 differential lipids, 6 of them being more abundant in melanoma cell lines. The distribution of the increased expression of PE ether lipids and molecules that could be either PC or PE ether lipids is distributed, since 2 out of 5 PE ether lipids and 7 out of 9 PC or PE ether lipids were representative of melanoma tissue. There were also 7 significant PGs that were able to differentiate between healthy and melanoma cells, presenting 5 of them increased levels for melanoma cells. In PSs however, their arrangement is more divided, as 2 PSs are more abundant in melanoma and other 2 species in melanocytes.

Table 28. List of lipids with significantly higher intensity in melanocytes (skin and nevus) (purple) **or in melanoma cells (primary and metastatic)** (light brown)**.** Analysis of the significance with t-test analysis (*<0.05; **<0.01; ***<0.001; ****<0.0001).

Lipid class	Lipid specie	t-test sign.
SM	Lipid 1	***
	lipid 3	***
	lipid 4	**
	lipid 5	**
	lipid 6	****
	lipid 7	***
	lipid 8	***
	lipid 10	****
	lipid 12	***
	lipid 13	**
	lipid 14	***
	lipid 15	****
	lipid 16	**
	lipid 17	***
DG	lipid 19	****
	lipid 20	***
PC	lipid 23	***
	lipid 25	***
	lipid 26	***
	lipid 27	***

	lipid 29	**
	lipid 31	***
	lipid 33	***
	lipid 34	**
	lipid 36	*
	lipid 37	**
	lipid 38	***
	lipid 39	****
	lipid 40	***
	lipid 42	***
	lipid 44	***
	lipid 45	****
	lipid 46	****
	lipid 47	***
	lipid 48	***
	lipid 50	****
	lipid 51	****
	lipid 52	****
PC ether	lipid 56	****
	lipid 58	****
	lipid 59	****
	lipid 60	***
	lipid 61	***
	lipid 62	**
	lipid 63	***
	lipid 65	***
	lipid 66	***
	lipid 67	***
	lipid 68	***
	lipid 69	***
	lipid 70	****
	lipid 71	***
	lipid 72	***
	lipid 73	***
	lipid 74	***
	lipid 75	***
	lipid 76	***
PE	lipid 78	***
	lipid 79	***
	lipid 80	**
	lipid 81	***
	lipid 83	***

	lipid 84	***
	lipid 85	***
PE ether	lipid 86	**
	lipid 88	**
	lipid 89	**
	lipid 90	****
	lipid 91	***
PC/PE	lipid 93	****
	lipid 94	****
	lipid 96	***
	lipid 98	****
	lipid 99	****
	lipid 100	**
	lipid 101	****
	lipid 102	****
	lipid 103	****
PC/PE ether	lipid 104	***
	lipid 105	***
	lipid 106	****
	lipid 107	***
	lipid 108	****
	lipid 109	****
	lipid 110	***
	lipid 112	****
	lipid 114	****
PS	lipid 116	***
	lipid 117	***
	lipid 118	****
	lipid 119	****
PG	lipid 120	**
	lipid 121	****
	lipid 122	***
	lipid 123	***
	lipid 124	****
	lipid 126	***
	lipid 127	***
PI	lipid 128	***
	lipid 129	**
	lipid 130	***
	lipid 131	***
	lipid 132	****
	lipid 133	***

	lipid 134	***
	lipid 135	**
	lipid 136	****
	lipid 137	***
	lipid 138	****
	lipid 139	***
	lipid 140	***
	lipid 141	**
	lipid 142	****
	lipid 143	****

The results obtained in the t-test analysis revealed that there are several lipid species with altered levels between melanocytes and malignant melanoma cells. Therefore, a multi-comparative t-test and post-hoc corrections were conducted. This analysis compared the intensity differences of each lipid specie in the four study groups in a paired analysis as follows: skin vs nevus melanocytes; skin melanocytes vs primary melanoma; nevus melanocytes vs primary melanoma; skin melanocytes vs metastatic melanoma; nevus melanocytes vs metastatic melanoma; primary vs metastatic melanoma. In order to choose the correct post-hoc correction for the significant comparisons in t-test, Levene's test was conducted. Here, if $p<0.05$, Games-Howell post-hoc correction was applied; however, when Levene's test significance was $p>0.05$, Bonferroni was conducted. The aim of these analyses was to identify the concrete lipid species that have differential distribution among the four different study groups. In this way, it could be differentiated the importance of each biomarker in the different stages of melanoma malignancy.

First, in the comparison of the two melanocytes groups, only 11 lipid molecules showed significantly different expression levels **(Table 29)**. Among these, 1 SM, 4 PCs and 1 alkyl-PC lipid were upregulated in skin melanocytes, whereas 1 PC(P/O), 1 PE, 2 PE plasmalogens and 1 PG showed higher levels in nevi. It is important to highlight that lipid 4, lipid 28, lipid 53, lipid 54, lipid 55, lipid 81 and lipid 120 that were identified as potential biomarkers in the comparison of skin and nevus melanocytes, are also identified as biomarkers when comparing skin melanocytes and primary melanoma cells. In addition to the previously mentioned molecules, lipid 57, lipid 63 and lipid 86 are also presented as biomarkers for differentiating skin melanocytes and metastatic melanoma cell lines. Markedly, 10 of the potential biomarkers identified when compared skin and nevus melanocytes are also identified as biomarkers when comparing skin melanocytes and melanoma cells, thus, these lipids might play a role in the malignification of nevus melanocytes.

Table 29. List of significant lipids with differential higher intensity in skin melanocytes (green) **or nevus melanocytes** (yellow)**.** Multi-comparative t-test with post-hoc correction: Games-Howell (Levene sign. p<0.05); Bonferroni (Levene sign p>0.05). Post-hoc significance: *<0.05; **<0.01; ***<0.001; ****<0.0001.

Lipid class	Lipid specie	Levene sign.	Post-Hoc	Sign. Post-hoc
SM	Lipid 4	0,1359	Bonferroni	*
PC	Lipid 28	0,1846	Bonferroni	**
	Lipid 53	0,1439	Bonferroni	**
	Lipid 54	0,1138	Bonferroni	**
	Lipid 55	0,2991	Bonferroni	***
PC ether	Lipid 57	0,0008	Games-Howell	*
	Lipid 63	0,0261	Games-Howell	*
PE	Lipid 81	0,0115	Games-Howell	*
PE ether	Lipid 86	0,0011	Games-Howell	*
	Lipid 87	0,6211	Bonferroni	*
PG	Lipid 120	0,0117	Games-Howell	*

In order to identify the lipids that are biomarkers for melanoma development, the lipid profiles of skin melanocytes and primary melanoma cell lines were compared **(Table 30)**. Here, 49 lipid molecules showed differential expression, with 32 species increasing their levels in the development of melanoma. Sphingomyelins are representative of healthy cells, with 8 molecules showing significant increased levels in skin melanocytes. On the other hand, the 7 PC ether lipids, 2 PC or PE, 3 PC or PE ether lipids, 1 PG and 6 PIs identified as biomarkers were associated with melanoma development. Besides, 1 out of 2 PS, 2 out of 6 PEs and 6 out of 14 PCs identified as biomarkers showed significant increased expression in skin melanocytes.

Table 30. List of significant lipids with differential higher intensity in skin melanocytes (green) **or primary melanomas** (red)**.** Multi-comparative t-test with post-hoc correction: Games-Howell (Levene sign. p<0.05); Bonferroni (Levene sign p>0.05). Post-hoc significance: *<0.05; **<0.01; ***<0.001; ****<0.0001.

Lipid class	Lipid specie	Levene sign.	Post-Hoc	Sign. Post-hoc
SM	Lipid 1	0,2645	Bonferroni	**
	Lipid 3	0,0088	Games-Howell	*
	Lipid 4	0,1359	Bonferroni	***
	Lipid 5	0,0013	Games-Howell	*
	Lipid 6	0,0047	Games-Howell	*
	Lipid 7	0,1646	Bonferroni	****
	Lipid 13	0,1068	Bonferroni	*
	Lipid 14	0,0218	Games-Howell	*
PC	Lipid 24	0,0445	Games-Howell	*
	Lipid 25	0,0233	Games-Howell	*
	Lipid 26	0,3967	Bonferroni	****
	Lipid 28	0,1846	Bonferroni	***
	Lipid 31	0,2915	Bonferroni	**
	Lipid 32	0,2474	Bonferroni	**
	Lipid 36	0,0626	Bonferroni	**
	Lipid 44	0,0102	Games-Howell	*
	Lipid 47	0,3639	Bonferroni	*
	Lipid 48	0,0001	Games-Howell	***
	Lipid 51	0,1439	Bonferroni	**
	Lipid 53	0,1138	Bonferroni	**
	Lipid 54	0,2991	Bonferroni	***

PC ether	Lipid 55	0,0273	Games-Howell	*
	Lipid 58	0,0071	Games-Howell	*
	Lipid 59	0,0747	Bonferroni	**
	Lipid 60	0,0353	Games-Howell	*
	Lipid 62	0,0059	Games-Howell	**
	Lipid 66	0,0035	Games-Howell	*
	Lipid 70	0,0372	Games-Howell	*
PE	Lipid 71	0,1892	Bonferroni	*
	Lipid 78	0,0155	Games-Howell	**
	Lipid 80	0,0115	Games-Howell	*
	Lipid 81	0,2781	Bonferroni	*
	Lipid 82	0,0958	Bonferroni	*
	Lipid 83	0,0516	Bonferroni	**
	Lipid 85	0,7750	Bonferroni	**
PC/PE	Lipid 96	0,0311	Games-Howell	*
PC/PE ether	Lipid 100	0,0028	Games-Howell	**
	Lipid 106	0,0217	Games-Howell	*
	Lipid 109	0,0088	Games-Howell	**
PS	Lipid 111	0,0734	Bonferroni	**
	Lipid 117	0,0060	Games-Howell	**
PG	Lipid 118	0,0117	Games-Howell	**
PI	Lipid 120	0,0269	Games-Howell	*
	Lipid 130	0,0119	Games-Howell	*
	Lipid 131	0,0001	Games-Howell	*
	Lipid 132	0,0245	Games-Howell	**
	Lipid 133	0,0016	Games-Howell	*
	Lipid 136	0,0039	Games-Howell	**

The lipidome comparison of nevi melanocytes and primary melanomas confirmed that there is a phenotypic transition between these two cell lines groups as 54 lipid species showed to have differential expression levels **(Table 31)**. 33 of the identified biomarkers are also present in the comparison of skin melanocytes and primary melanomas, therefore, the other 21 gain significant insight in the development of melanoma from nevus melanocytes. As well as in the remaining comparisons, the 15 SMs detected are representative of healthy cells, while the 9 PIs, 1 DG, 4 PEs and 8 PC or PE ether lipids were associated with melanoma cells.

Table 31. List of significant lipids with differential higher intensity in nevus melanocytes (yellow) **or primary melanomas** (red)**.** Multi-comparative t-test with post-hoc correction: Games-Howell (Levene sign. p<0.05); Bonferroni (Levene sign p>0.05). Post-hoc significance: *<0.05; **<0.01; ***<0.001; ****<0.0001.

Lipid class	Lipid specie	Levene sign.	Post-Hoc	Sign. Post-hoc
SM	Lipid 1	0,2645	Bonferroni	***
	Lipid 2	0,3254	Bonferroni	*
	Lipid 3	0,0088	Games-Howell	**
	Lipid 4	0,1359	Bonferroni	*
	Lipid 5	0,0013	Games-Howell	***
	Lipid 6	0,0047	Games-Howell	**
	Lipid 7	0,1646	Bonferroni	****
	Lipid 8	0,0173	Games-Howell	**
	Lipid 9	0,7665	Bonferroni	*
	Lipid 12	0,0707	Bonferroni	**
	Lipid 13	0,1068	Bonferroni	****
	Lipid 14	0,0218	Games-Howell	*
	Lipid 15	0,2615	Bonferroni	***
	Lipid 16	0,0146	Games-Howell	****
	Lipid 17	0,0028	Games-Howell	****
DG	Lipid 20	0,0020	Games-Howell	*
PC	Lipid 25	0,0233	Games-Howell	*
	Lipid 26	0,3967	Bonferroni	***
	Lipid 31	0,2915	Bonferroni	**
	Lipid 32	0,0045	Games-Howell	**
	Lipid 33	0,0503	Bonferroni	*
	Lipid 37	0,2309	Bonferroni	****
	Lipid 38	0,0626	Bonferroni	**
	Lipid 44	0,0039	Games-Howell	**
PC ether	Lipid 45	0,0273	Games-Howell	*
	Lipid 58	0,0071	Games-Howell	*
	Lipid 59	0,0747	Bonferroni	***
	Lipid 60	0,0059	Games-Howell	**
	Lipid 62	0,2523	Bonferroni	*
PE	Lipid 63	0,0155	Games-Howell	**
	Lipid 80	0,0958	Bonferroni	*
	Lipid 83	0,1021	Bonferroni	*
	Lipid 84	0,0516	Bonferroni	*
PE ether	Lipid 85	0,1065	Bonferroni	****
	Lipid 88	0,0633	Bonferroni	**
	Lipid 91	0,1910	Bonferroni	**
PC/PE	Lipid 92	0,7750	Bonferroni	*
	Lipid 96	0,0311	Games-Howell	*
PC/PE ether	Lipid 100	0,0028	Games-Howell	**
	Lipid 107	0,0217	Games-Howell	*
	Lipid 109	0,3890	Bonferroni	***
PS	Lipid 114	0,0734	Bonferroni	**
PG	Lipid 117	0,0117	Games-Howell	*
	Lipid 120	0,1215	Bonferroni	**
	Lipid 121	0,0196	Games-Howell	*
	Lipid 123	0,0004	Games-Howell	*
PI	Lipid 124	0,0269	Games-Howell	*
	Lipid 130	0,0119	Games-Howell	*
	Lipid 131	0,0001	Games-Howell	*

	Lipid 132	0,0245	Games-Howell	**
	Lipid 133	0,0201	Games-Howell	*
	Lipid 134	0,0156	Games-Howell	**
	Lipid 135	0,0016	Games-Howell	*
	Lipid 136	0,0039	Games-Howell	**
	Lipid 139	0,0887	Bonferroni	*

As it was expected, the comparison between skin melanocytes and metastatic melanoma cell lines showed that many lipids had the capacity to discriminate between these two groups. In fact, 82 lipid species were observed to have different expression levels, 61 of them being upregulated in melanoma cells **(Table 32)**. In addition, the comparative analysis of nevi melanocytes versus metastatic melanoma cell lines showed that 81 lipid molecules **(Table 33)** had significantly different levels.

Table 32. List of significant lipids with differential higher intensity in skin melanocytes (green) **or metastatic melanomas** (blue)**.** Multi-comparative t-test with post-hoc correction: Games-Howell (Levene sign. p<0.05); Bonferroni (Levene sign p>0.05). Post-hoc significance: *<0.05; **<0.01; ***<0.001; ****<0.0001.

Lipid class	Lipid specie	Levene sign.	Post-Hoc	Sign. Post-hoc
SM	Lipid 1	0,2645	Bonferroni	**
	Lipid 4	0,1359	Bonferroni	***
	Lipid 5	0,0013	Games-Howell	*
	Lipid 6	0,0047	Games-Howell	*
	Lipid 7	0,1646	Bonferroni	***
HexCer	Lipid 18	0,9885	Bonferroni	*
DG	Lipid 19	0,0023	Games-Howell	**
	Lipid 20	0,0020	Games-Howell	***
PC	Lipid 23	0,0018	Games-Howell	*
	Lipid 24	0,0445	Games-Howell	*
	Lipid 25	0,0233	Games-Howell	*
	Lipid 26	0,3967	Bonferroni	***
	Lipid 27	0,0004	Games-Howell	**
	Lipid 28	0,1846	Bonferroni	****
	Lipid 30	0,0509	Bonferroni	**
	Lipid 31	0,2915	Bonferroni	***
	Lipid 36	0,2474	Bonferroni	**
	Lipid 39	0,0000	Games-Howell	***
	Lipid 40	0,0004	Games-Howell	*
	Lipid 41	0,0084	Games-Howell	*
	Lipid 44	0,0626	Bonferroni	***
	Lipid 47	0,0102	Games-Howell	**
	Lipid 48	0,3639	Bonferroni	**
	Lipid 51	0,0001	Games-Howell	**
	Lipid 53	0,1439	Bonferroni	***
	Lipid 54	0,1138	Bonferroni	**
	Lipid 55	0,2991	Bonferroni	**
PC ether	Lipid 57	0,0008	Games-Howell	*
	Lipid 58	0,0273	Games-Howell	*
	Lipid 59	0,0071	Games-Howell	**
	Lipid 60	0,0747	Bonferroni	**
	Lipid 61	0,0231	Games-Howell	**
	Lipid 62	0,0353	Games-Howell	***

	Lipid 64	0,9465	Bonferroni	*
	Lipid 66	0,0059	Games-Howell	***
	Lipid 67	0,2523	Bonferroni	*
	Lipid 68	0,0051	Games-Howell	*
	Lipid 70	0,0035	Games-Howell	*
	Lipid 71	0,0372	Games-Howell	*
	Lipid 72	0,0261	Games-Howell	*
	Lipid 73	0,0024	Games-Howell	*
	Lipid 74	0,0306	Games-Howell	*
	Lipid 75	0,0017	Games-Howell	*
	Lipid 76	0,0008	Games-Howell	*
PE	Lipid 77	0,0375	Games-Howell	*
	Lipid 78	0,1892	Bonferroni	**
	Lipid 80	0,0155	Games-Howell	**
	Lipid 81	0,0115	Games-Howell	****
	Lipid 82	0,2781	Bonferroni	**
	Lipid 83	0,0958	Bonferroni	**
	Lipid 84	0,0516	Bonferroni	*
PE ether	Lipid 86	0,0011	Games-Howell	**
	Lipid 88	0,1065	Bonferroni	*
PC/PE	Lipid 95	0,0297	Games-Howell	***
	Lipid 96	0,7750	Bonferroni	**
	Lipid 97	0,0245	Games-Howell	*
	Lipid 98	0,0030	Games-Howell	**
	Lipid 99	0,9568	Bonferroni	**
	Lipid 100	0,0311	Games-Howell	***
	Lipid 103	0,0000	Games-Howell	***
PC/PE ether	Lipid 107	0,0028	Games-Howell	**
	Lipid 109	0,0217	Games-Howell	*
	Lipid 110	0,0069	Games-Howell	**
	Lipid 111	0,0088	Games-Howell	**
PS	Lipid 115	0,0005	Games-Howell	***
	Lipid 117	0,0734	Bonferroni	*
	Lipid 118	0,0060	Games-Howell	****
	Lipid 119	0,0407	Games-Howell	**
PG	Lipid 120	0,0117	Games-Howell	***
	Lipid 125	0,0086	Games-Howell	***
	Lipid 127	0,0151	Games-Howell	**
PI	Lipid 130	0,0269	Games-Howell	**
	Lipid 131	0,0119	Games-Howell	**
	Lipid 132	0,0001	Games-Howell	*
	Lipid 133	0,0245	Games-Howell	**
	Lipid 136	0,0016	Games-Howell	****
	Lipid 137	0,0852	Bonferroni	**
	Lipid 138	0,0563	Bonferroni	*
	Lipid 139	0,0039	Games-Howell	****
	Lipid 140	0,0017	Games-Howell	****
	Lipid 141	0,0164	Games-Howell	****
	Lipid 142	0,0887	Bonferroni	*

Table 33. List of significant lipids with differential higher intensity in nevus melanocytes (yellow) **or metastatic melanoma** (blue)**.** Multi-comparative t-test with post-hoc correction: Games-Howell (Levene sign. p<0.05); Bonferroni (Levene sign p>0.05). Post-hoc significance: *<0.05; **<0.01; ***<0.001; ****<0.0001.

Lipid class	Lipid specie	Levene sign.	Post-Hoc	Sign. Post-hoc
SM	Lipid 1	0,2645	Bonferroni	***
	Lipid 3	0,0088	Games-Howell	*
	Lipid 5	0,0013	Games-Howell	***
	Lipid 7	0,1646	Bonferroni	****
	Lipid 8	0,0173	Games-Howell	*
	Lipid 12	0,0707	Bonferroni	***
	Lipid 13	0,1068	Bonferroni	****
	Lipid 14	0,0218	Games-Howell	*
	Lipid 15	0,2615	Bonferroni	**
	Lipid 16	0,0146	Games-Howell	***
	Lipid 17	0,0028	Games-Howell	****
DG	Lipid 19	0,0023	Games-Howell	**
	Lipid 20	0,0020	Games-Howell	****
TG	Lipid 21	0,1286	Bonferroni	*
PC	Lipid 25	0,0233	Games-Howell	****
	Lipid 26	0,3967	Bonferroni	***
	Lipid 27	0,0004	Games-Howell	*
	Lipid 29	0,0109	Games-Howell	***
	Lipid 31	0,2915	Bonferroni	**
	Lipid 33	0,0045	Games-Howell	**
	Lipid 34	0,1777	Bonferroni	*
	Lipid 37	0,0503	Bonferroni	**
	Lipid 38	0,2309	Bonferroni	****
	Lipid 39	0,0000	Games-Howell	**
	Lipid 40	0,0084	Games-Howell	**
	Lipid 43	0,4723	Bonferroni	*
	Lipid 44	0,0626	Bonferroni	****
	Lipid 45	0,0039	Games-Howell	**
	Lipid 47	0,0102	Games-Howell	**
	Lipid 49	0,0222	Games-Howell	*
	Lipid 51	0,0001	Games-Howell	**
	Lipid 52	0,0576	Bonferroni	**
PC ether	Lipid 58	0,0273	Games-Howell	*
	Lipid 59	0,0071	Games-Howell	**
	Lipid 60	0,0747	Bonferroni	****
	Lipid 61	0,0231	Games-Howell	**
	Lipid 62	0,0353	Games-Howell	***
	Lipid 63	0,1030	Bonferroni	**
	Lipid 66	0,0059	Games-Howell	***
	Lipid 67	0,2523	Bonferroni	***
	Lipid 68	0,0051	Games-Howell	*
	Lipid 73	0,0024	Games-Howell	*
	Lipid 74	0,0306	Games-Howell	*
	Lipid 75	0,0017	Games-Howell	*
	Lipid 76	0,0008	Games-Howell	*
PE	Lipid 80	0,0155	Games-Howell	**
	Lipid 83	0,0958	Bonferroni	**
	Lipid 84	0,0516	Bonferroni	*
PE ether	Lipid 86	0,0011	Games-Howell	*

	Lipid 88	0,1065	Bonferroni	****
	Lipid 90	0,1225	Bonferroni	**
PC/PE	Lipid 94	0,3676	Bonferroni	*
	Lipid 96	0,7750	Bonferroni	*
	Lipid 98	0,0030	Games-Howell	*
	Lipid 99	0,9568	Bonferroni	***
	Lipid 100	0,0311	Games-Howell	***
	Lipid 103	0,0000	Games-Howell	**
PC/PE ether	Lipid 104	0,0016	Games-Howell	*
	Lipid 105	0,0409	Games-Howell	**
	Lipid 106	0,0028	Games-Howell	***
	Lipid 109	0,0217	Games-Howell	*
	Lipid 110	0,0069	Games-Howell	**
	Lipid 114	0,3890	Bonferroni	**
PS	Lipid 117	0,0734	Bonferroni	**
	Lipid 118	0,0060	Games-Howell	*
	Lipid 119	0,0407	Games-Howell	*
PG	Lipid 120	0,0117	Games-Howell	**
	Lipid 123	0,0196	Games-Howell	*
	Lipid 124	0,0004	Games-Howell	*
	Lipid 127	0,0151	Games-Howell	*
PI	Lipid 130	0,0269	Games-Howell	**
	Lipid 131	0,0119	Games-Howell	**
	Lipid 132	0,0001	Games-Howell	*
	Lipid 133	0,0245	Games-Howell	*
	Lipid 134	0,0201	Games-Howell	****
	Lipid 135	0,0156	Games-Howell	*
	Lipid 136	0,0016	Games-Howell	***
	Lipid 137	0,0852	Bonferroni	**
	Lipid 138	0,0563	Bonferroni	**
	Lipid 139	0,0039	Games-Howell	**
	Lipid 140	0,0017	Games-Howell	****
	Lipid 141	0,0164	Games-Howell	****
	Lipid 142	0,0887	Bonferroni	**

Finally, primary and metastatic melanoma cells were confronted to identify the lipids that can contribute to the metastatic progression of the malignancy. Here, only three lipid molecules were identified **(Table 34)**. Indeed, levels of lipid 10, lipid 11 and lipid 22 increased with the metastatic progression of the melanoma cells.

Table 34. List of significant lipids with differential higher intensity in primary or metastatic melanoma (blue)**.** Multi-comparative t-test with post-hoc correction: Games-Howell (Levene sign. p<0.05); Bonferroni (Levene sign p>0.05). Post-hoc significance: *<0.05; **<0.01; ***<0.001; ****<0.0001.

Lipid class	Lipid specie	Levene sign.	Post-Hoc	Sign. Post-hoc
SM	Lipid 10	0,0010	Games-Howell	*
	Lipid 11	0,0001	Games-Howell	*
TG	Lipid 22	0,0009	Games-Howell	*

3. Phospholipase D2 enhances melanoma progression and metastatic behavior

3.1. PLD2 enzyme is upregulated in melanoma cells

Several members of the phospholipase family of enzymes have been related to some malignancies such as breast and colon tumors, among others[132]. However, no clear relation to melanoma has been described yet. Nonetheless, the lipid content modifications described in the previous sections suggest that these enzymes might also be affected, as many of the significantly altered GPLs are substrates of these enzymes.

In order to study the connection between these proteins and melanoma, the expression levels of phospholipase A_2, C, D1 and D2 were analyzed by western blot. This was performed in three different skin melanocyte cell lines (HEMn-LP, Hemn-MP, HEMn-DP), five primary melanomas (Mel-Ho, G361, Sk-Mel-28, ME4405, A375), and nine metastatic melanomas of different origins (Sk-Mel-3, VMM1, HT144, Colo-800, A2058, RPMI-7951, Hs294t, WM-266-4, Sk-Mel-2). The comparison of the protein levels obtained for each enzyme **(Fig. 55)** suggested that skin melanocytes have high levels of PLA_2, higher than primary melanomas. However, some of the metastatic melanomas, namely HT144, Colo-800 and RPMI-7951, expressed greater levels of this protein, although it was cell line specific. PLC on its side was evenly expressed in all the cell lines studied, and no marked differences were observed among the groups. Concerning PLD enzymes, there are two main members, PLD1 and PLD2. Although metastatic melanoma cells showed high PLD1 expression levels, there was no evident difference between skin melanocytes and primary melanoma, being cell line specific. In fact, HEMn-MP cell line showed higher expression levels than all the primary melanomas but Mel-Ho. However, the western blot results obtained for PLD2 enzyme suggested that skin melanocytes have very low expression of this enzyme, which increases accordingly to melanoma progression. Moreover, these results were confirmed by immunofluorescence. As it can be seen in **Figure 56**, the expression of PLD2 in melanocytes was undetectable by immunofluorescence, while A375 and Mel-Ho primary melanoma cell lines, and Hs294t and Colo-800 metastatic cells showed higher PLD2 expression than melanocytes, what correlates with western blot results.

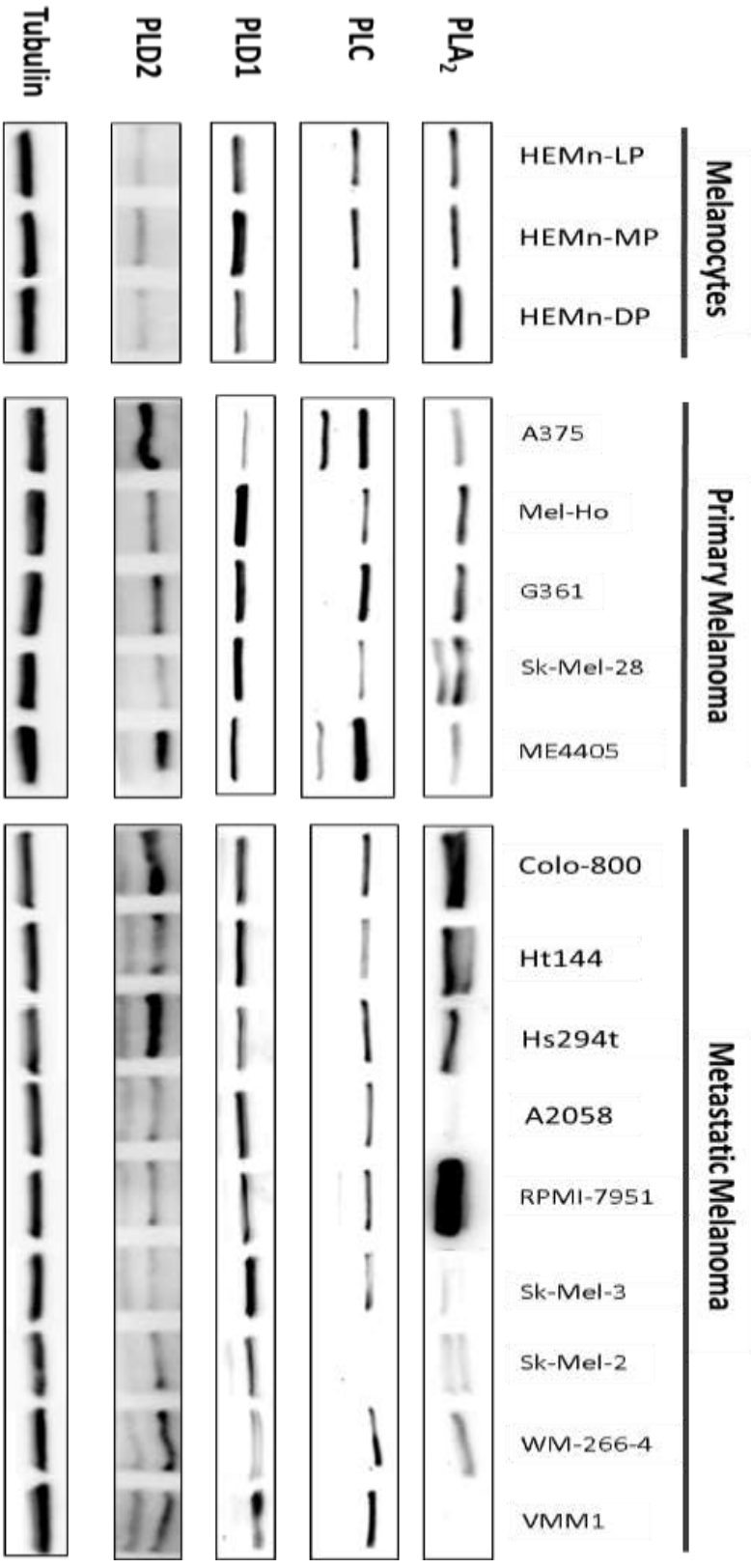


Figure 54. Protein expression pattern of different phospholipases (PLA_2, PLC, PLD1, PLD2) in 3 skin melanocytes, 5 primary melanomas and 9 metastatic melanoma cell lines assessed by Western blot. Tubulin expression was used as loading control. Figure is representative of four independent studies.

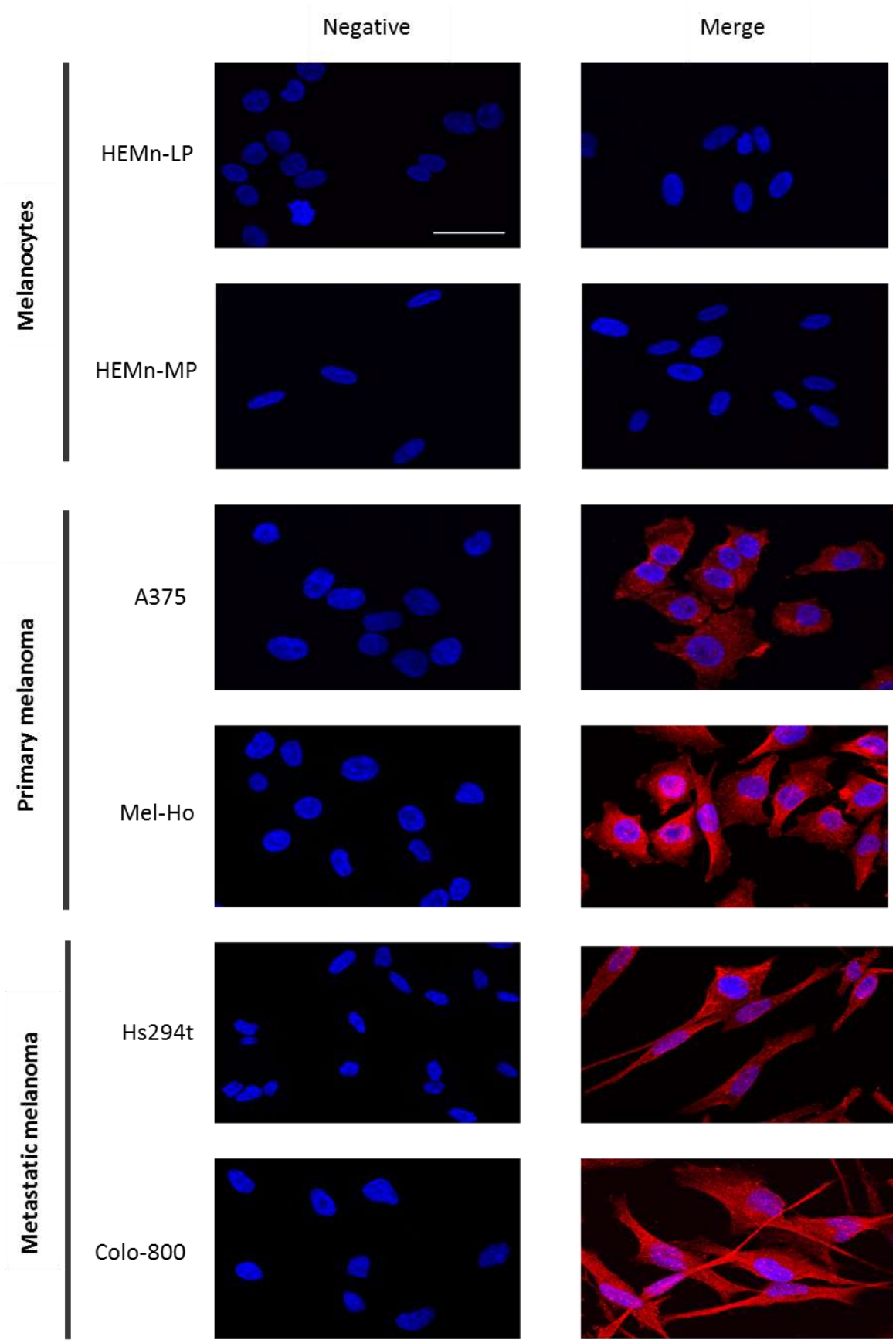


Figure 55. Identification of PLD2 expression pattern in skin melanocytes, primary melanoma and metastatic melanoma cell lines by immunofluorescence. PLD2 was stained in red. Nuclei were stained in blue with hoechst. Scale bar, 50-µm. Original magnification, x63. These images represent at least three different experiments.

Aside protein expression, PLD enzymatic activity was also found altered in other cancers. For that reason, this was studied in HEMn-LP, HEMn-MP and HEMn-DP skin melanocytes, A375, Sk-Mel-28 and Mel-Ho primary melanomas, and Colo-800 and A2058 metastatic melanoma cell lines. The results in **Figure 57** show that there was increased PLD enzymatic activity that correlated with melanoma progression. Indeed, skin melanocytes showed lower activity than primary melanomas overall, although the activity of HEMn-LP and primary melanomas was similar. Nonetheless, there was a significant increase in PLD activity of metastatic melanoma cells, thereby supporting that PLD enzymes play role in melanoma, especially in the metastasis.

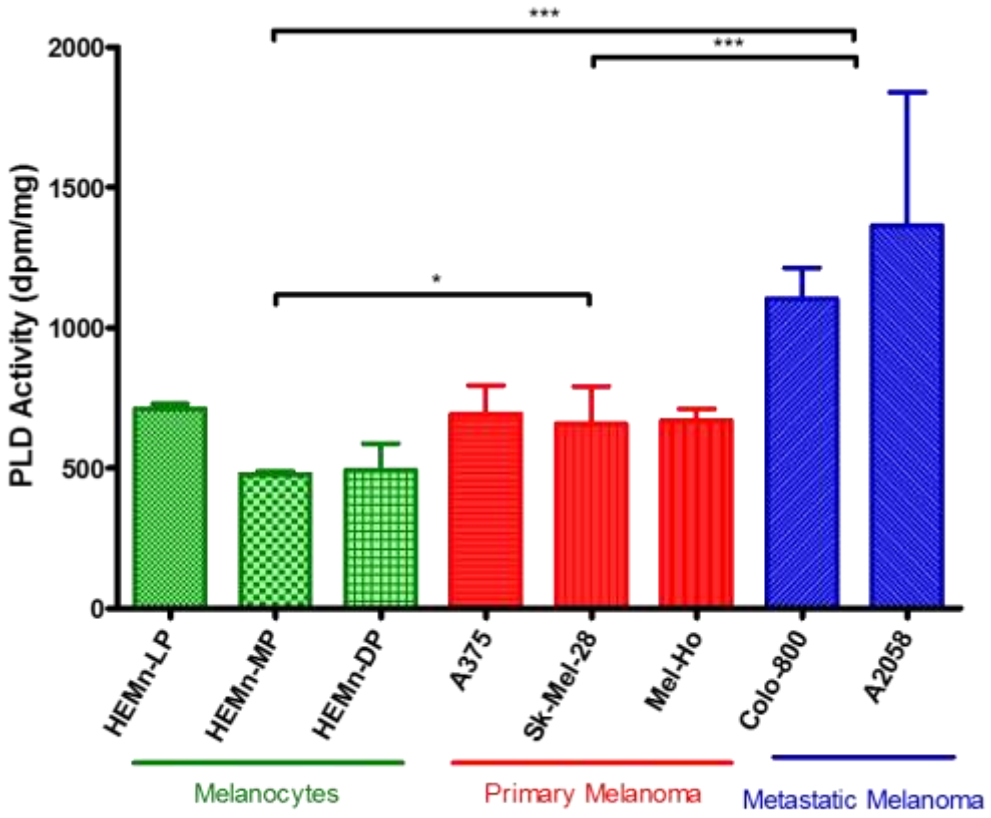


Figure 56. PLD enzymatic activity for melanocytes (green)**, primary melanomas** (red) **and metastatic melanomas** (blue). PLD enzymes activity increases with melanoma progression. PLD activity is expressed as dpm/mg of protein for each sample. The results represent three independent experiments. Data are given as mean ± SEM. Statistical analyses: unpaired t-test (p*<0.05; **<0.01; ***<0.001).

3.2. PLD2 involvement in the carcinogenic process

In order to determine the implication of this enzyme in the different steps of the carcinogenic process, transient genetic overexpression and silencing of PLD2 was carried out in primary melanoma cells (A375 and Mel-Ho) and metastatic cells (Colo-800). Western blot analyses were conducted to confirm the correct transfection of the cells. Both overexpression and silencing transfections have a control condition, so the expression levels of the transfected cells was related to each control condition. The western blot results confirmed the increased PLD2 expression in the overexpressed cells, whereas a decreased signal was observed in the silenced cells **(Fig.58 (A))**. Furthermore, as this is an enzyme, in addition to protein expression the changes in the enzymatic activity are essential. Thus, the analysis of PLD activity demonstrated that the lipase activity was also altered in the transfected cells **(Fig. 58 (B))**. The results obtained in the experiments carried out in the transfected cells were normalized to the transfection control condition; this is, the control was set in 1, so the cells with higher activity than the control would be represented above 1, whereas the cells with decreased activity would have an activity lower than 1. In our study, three different experiments of three different transfections of each cell line showed a mean of 1.87 activity in overexpressed A375, 1.54 in Mel-Ho, and 1.4 in Colo-800. Concerning the downregulated cells, A375 showed a 0.52 activity, Mel-Ho a 0.82, and Colo-800 a 0.68.

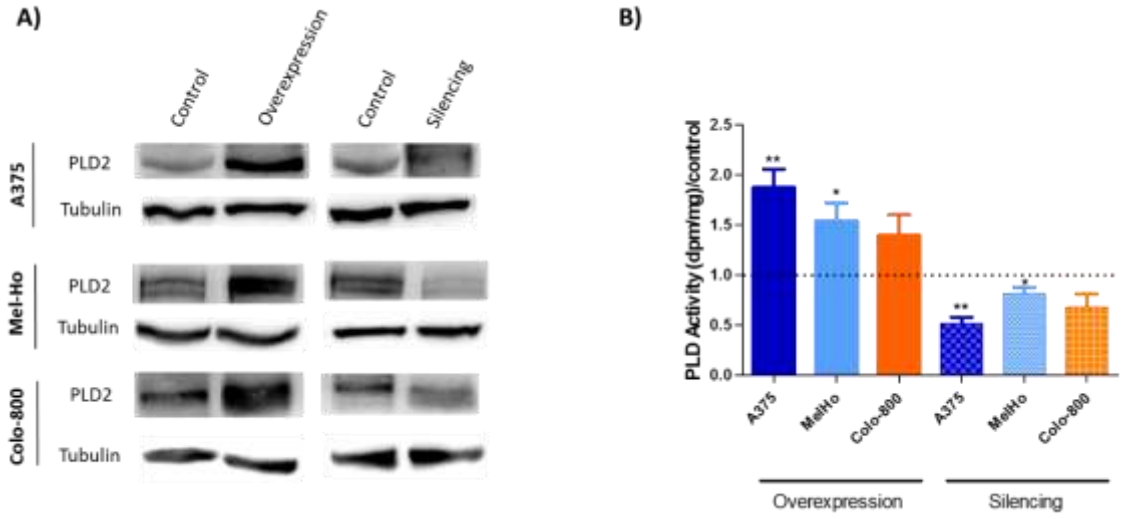


Figure 57. PLD2 protein expression (A) and PLD enzymatic activity (B) studies in PLD2 overexpressed and silenced cell lines. (A) The images are representative of three different PLD2 transfections and western blot analysis. (B) PLD enzymatic activity results are expressed as dpm/mg of protein of each sample, related to the transfection control values. Results are representative of three different transfections and activity experiments. Data are given as mean ± SEM. Statistical analyses: unpaired t-test (p*<0.05; **<0.01; ***<0.001).

The western blot and PLD activity experiments demonstrated the correct transfection of the cells. Thus, the next step was to study the effect of these genetic alterations in cell proliferation, migration and invasion. Although the effects are cell line specific, there is a clear tendency of increased proliferation, migration and invasion in PLD2 overexpressed cell lines, while these processes are downregulated in PLD2 silenced cells **(Fig. 59)**. Indeed, cell proliferation was markedly increased in A375 overexpressed cells (1.51) and Colo-800 (1.68), while overexpressed Mel-Ho cells presented a slight increase (1.06). In the PLD2 downregulated cells however, all the cell lines reduced their proliferation: A375 (0.55), Mel-Ho (0.8) and Colo-800 (0.79). Concerning migration, both overexpressed primary melanoma cells presented similar increments, A375 (1.39) and Mel-Ho (1.31), while this process was very significantly increased in metastatic cells (2.13). In the silenced cells however, the results are more similar among the cell lines, with a clear decrease in cell migration: A375 (0.66), Mel-Ho (0.53) and Colo-800 (0.54). Last, cell invasion analyses showed that this was notably increased in primary melanoma, A375 (1.68) and Mel-Ho (1.71), compared to metastatic cells (1.43). However, in the silenced cells the results are more similar, although primary melanoma cells are still more affected, A375 (0.48), Mel-Ho (0.53), Colo-800 (0.60).

(A)

	PLD activity		Proliferation		Migration		Invasion	
	OE	Si	OE	Si	OE	Si	OE	Si
A375	1.87	0.52	1.51	0.55	1.39	0.66	1.68	0.48
Mel-Ho	1.54	0.82	1.06	0.80	1.31	0.53	1.71	0.53
Colo-800	1.40	0.68	1.68	0.79	2.13	0.54	1.43	0.60

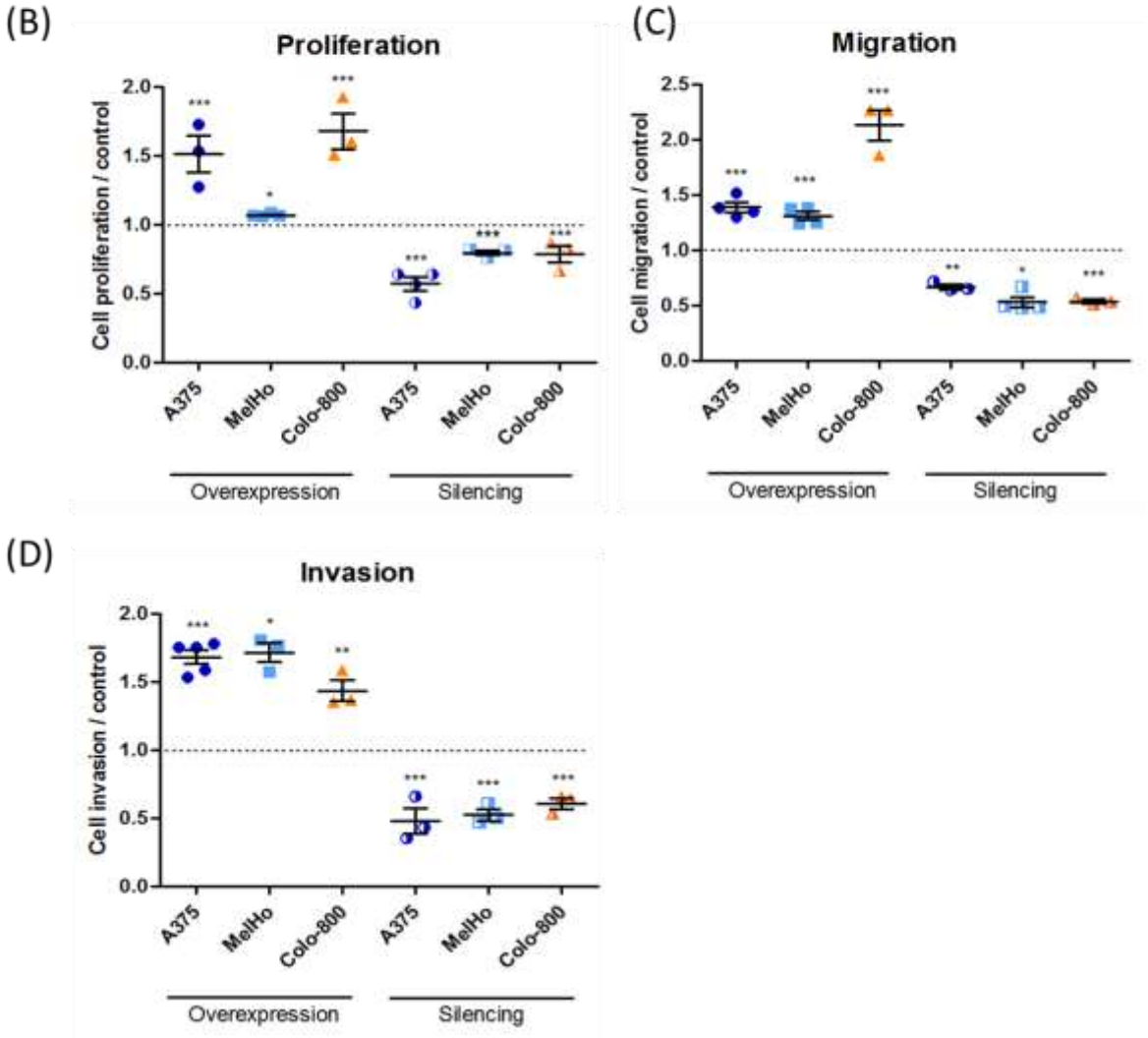


Figure 58. (A) Summary of the results obtained for PLD2 overexpressed and silenced melanoma cell lines in PLD activity, cell proliferation, migration and invasion. Graphical visualization of cell proliferation (B), migration (C) and invasion (D). studies in PLD2 overexpressed and silenced cell lines. Results of each experiment are expressed related to the values obtained for the transfection control. Data are given as mean ± SEM of at least three experiments of different transfections. Statistical analyses: Unpaired t-test or non-parametric Mann-Whitney test (p*<0.05; **<0.01; ***<0.001).

DISCUSSION

1. Global lipid profiling of human melanocytes and melanoma cell lines

The incidence rate of cutaneous melanoma has steadily increased in recent decades. Indeed, it is the seventh most common tumor worldwide and is responsible for 80% of skin cancer deaths[1]. However, the mortality rate has not followed the same trend as incidence and has remained stable for the last 20 years. This stabilization is owed to improvements in awareness, early diagnosis, and treatments[4]. It is worth noting that since 2011, the FDA has approved 10 different treatment options, increasing the one-year overall survival from 16-30% to 50-70%. The outcome of the patient is directly related to the diagnosis stage, and although the recovery rate is close to 100% in early-diagnosed patients, there is no effective therapy for metastatic melanoma[29]. The diagnosis is made based on clinical screening and subsequent histopathological confirmation. However, there are not completely efficacious biomarkers for cutaneous melanoma. Therefore, it stands to reason that new biomarkers that can help in the early diagnosis and prognosis or that can be therapeutic targets must be discovered.

Recently, the study of the lipid content of cells, this is the lipidome, and their metabolism in cancer has emerged as a very promising research field. As a matter of fact, deregulated cellular energetics and metabolic adaptation are acquired features of cancer cells[42]. A large body of work demonstrates that cancer cells adapt their lipid content and metabolism to support the malignant phenotype that enables their survival, proliferation and growth in the harsh tumor microenvironment. Lipid metabolism rewiring in cancer cells in particular is defined by increased *de novo* biosynthesis of lipids together with increased oxidation of lipids. These alterations generate aberrant cell signaling pathways, disrupted energy production, structural alterations in cell membranes, and distinct gene and protein expression, what is translated into deregulated cell growth, proliferation, death, and resistance to drugs and chemotherapy[49], for instance. Furthermore, the aberrant lipid metabolism of cancer cells has been strongly related to the metastatic dissemination of the cells[114]. This has been studied especially in breast, colon, lung and prostate cancers[49,96,152], among others, but little is known about lipidome and metabolism alterations in melanoma cells.

Therefore, a comparative study of the lipidome of melanocytes and melanoma cells was outlined, with the aim to identify the global lipid fingerprint of melanoma and melanocyte cells, and the particular lipid species that account for the differentiation of the lipid content of normal and tumor cells in melanoma, as well as the biological implications of those changes. For this, the lipidome of skin and nevus melanocytes cell lines, and primary and metastatic melanoma cell lines was studied using different lipidomics approaches, which are used to ascertain and classify tumor cells and tissues as they characterize the lipid content of a sample and the interactions of these lipids with other lipids, proteins, metabolites and genetic material[96].

Lipidomic analyses are mainly grounded on mass spectrometry techniques. ESI (electrospray ionization) and MALDI (matrix-assisted laser desorption ionization) are the most important ionization sources for the study of biomolecules in mass spectrometry[145]. MALDI-MS is frequently employed for biomarker discovery, and is used for direct lipid identification with no need of previous lipid extraction. MALDI-MS needs the deposition of a low-molecular weight organic matrix that absorbs the laser radiation and emits molecular ions. The advantages of using MALDI ionization is its lack of necessity for labeling, high sensitivity, high throughout and molecular-specificity[105]. However, as there is gentle ionization in MALDI, only general information about the structure of the lipid is achieved, with no specification of the particular side-chains. To overcome this, ESI tandem MS/MS can be used, as it is frequently coupled to liquid chromatography for a pre-separation of the lipid families of the lipid extracts to achieve a

better identification of the molecules. Moreover, as it uses a tandem MS/MS strategy, the precursor ion and its subsequent fragments are detected, making up the structure of the precursor molecule. Indeed, ESI is the most used ionization method for lipids[105].

The first strategy of this work was to assess whether there were notifiable differences in the lipid composition of lipid extracts of skin melanocytes cell lines, nevus melanocytes, primary melanoma cell lines and metastatic melanoma cell lines, employing a very commonly used MS strategy for the study of lipids: UHPLC-ESI-MS/MS. The results obtained were examined by non-supervised PCA and supervised PLS-DA analysis, and although the lipid phenotype of skin vs nevus melanocytes and primary vs metastatic melanoma were not different enough to be separated by these analytical approaches, it was confirmed that non-malignant (skin + nevus melanocytes) and malignant cells (primary + metastatic melanoma) present different lipid content. Hence, the lipidomic approach employed is appropriate for the study of the aberrant lipidome that tumor cells exhibit.

A deeper view of the results revealed that the different lipid families have altered intensities within the study groups, in order to support the malignant transformation and survival of cancer cells. A general overview of our results shows an increased intensity in choline bearing lipids in metastatic melanoma cells compared to skin melanocytes. This is a common feature for many tumors as it has been identified as well in colorectal[153], breast, brain, lung, ovary and prostate cancers[154]. The main function of choline glycerolipids is structural, as they represent 50% of the membrane lipids, and besides this, they are also signaling molecules, as they are traduced into mitogenic signals. The increased intensities of this family have been linked to various alterations in their metabolism in a wide variety of cancers. First, increased choline uptake is a common trait of cancer cells, and for this, choline transporters are needed, which have been found very highly expressed in melanoma cells[127]. According to the biosynthesis, the first enzyme of the Kennedy pathway, the choline kinase, has been detected aberrantly activated in lung, colon, breast, prostate, cervix and ovary tumor cells in part due to alterations in Ras and PI3K signaling cascades[55], which are the main pathways in melanoma and are constitutively activated. Markedly, the intensities of choline lipids are used to monitor chemotherapy efficacy, as decreased signals are linked to good therapeutic response[123]. Conversely, the second most common glycerophospholipid family of the membranes, in our study phosphatidylethanolamine lipid species presented decreased intensities in melanoma cells and nevus melanocytes compared to skin melanocytes, although there was great variance in the levels among the cell lines of each study group. Indeed, there is controversy in bibliography and the results are cancer type specific. For instance, similar to our results, PE levels were downregulated in clear cell renal cell carcinoma and upon the exposure of these malignant cells to external ethanolamine their proliferation was attenuated, while no effect was detected in normal cells[155].

Membrane homeostasis is critically dependent on its lipid composition and slight changes in its lipid content alters membrane integrity. Phospholipids comprise the bulk of membrane lipids, and both the head group and acyl side-chains determine the biophysical properties of the membranes. PCs, that are the most represented lipids in the bilayers, have a cylindrical molecular shape due to the size of their head group. This enables them to self-assemble tightly forming a stable membrane. Moreover, PCs usually have a cis-unsaturated fatty acyl chain, lowering their packaging and increasing the fluidity of the membrane. By contrast, PEs have a conical shape because of the small size of their head group, what imposes a negative stress curvature in the membrane. This creates lipid-packaging defects and facilitates membrane fusion, fission and budding[156,157]. Hence, the ratio of cylindrical/conical lipids is critical to

maintain the integrity and proper function of the bilayers, where PCs are mainly localized to the external leaflet whereas PEs are located predominantly on the cytoplasmic leaflet[156,158]. The ratios obtained for PC/PE in our work show that this is increased upon melanoma development, as it goes from 1 in skin melanocytes to 1.5 in primary melanoma and 1.7 in metastatic melanoma **(Table 19)**, what is translated into increased fluidity and decreased permeability in melanoma cells membranes[159,160]. This is a characteristic observed in various solid tumors, as it favors the proliferative and invasive phenotype. Indeed, the metastatic potential of tumor cells is significantly correlated with increased membrane fluidity[161]. Furthermore, effective chemotherapeutic treatment has been linked to generating a drop in membrane fluidity and membrane permeability enlargement, while resistant cells show increased membrane rigidity, and hence less permeability, resulting in poor drug penetration[162]. The augmented choline uptake and *de novo* choline lipids biosynthesis observed in many cancers might not be sufficient to supply all the PC required by the highly proliferative cancer cells, so, melanoma cells might increase the expression and activity of PEMT to cover the augmented choline needs. PEMT is an integral membrane enzyme that directly transforms PEs into PCs following three consecutive methylations. Regarding other tumor types, data is inconsistent; while its activity is significantly dismissed in liver and breast cancer, with the subsequent increase in ethanolamine lipids in these cancers[127], opposite results were observed in a study in lung cancer cells, presenting data consistent with our results[153]. In general, aggressive tumors have been linked to increased PC/PE ratio and augmented PEMT activity[153]. Our results suggest that this enzyme could gain activity upon melanoma development, since the ratio between the concrete PC/PE lipids that share the same acyl-chains is increased in metastatic melanomas in all the cases but one: PC(16:0/20:4)-PE(18:1/18:3) or PE(16:0/20:4).

Table 35. PC/PE ratio of the intensities obtained for the different PC and PE species that have the same acyl-chains, related to control group (skin melanocytes =1).

	PC/PE ratio related to skin melanocytes			
	Skin melanocytes	Nevus melanocytes	Primary melanoma	Metastatic melanoma
PC(16:0/16:1)/ PC(14:0/18:1)	1.00	1.09	1.61	2.26
PE(16:0/16:1)				
PC(16:0/18:2)	1.00	2.39	0.73	1.32
PE(34:2)				
PC(16:0/18:1)	1.00	1.32	1.43	1.65
PE(34:1)				
PC(18:0/18:1)	1.00	2.46	1.26	2.10
PE(18:0/18:1)				
PC(38:3)	1.00	2.12	1.62	2.25
PE(18:1/20:2)/ PE(18:0/20:3)				
PC(18:0/20:2)	1.00	1.28	3.26	4.33
PE(18:1/20:1)/ PE(18:0/20:2)				
PC(40:5)	1.00	1.97	1.05	1.23
PE(18:1/22:4)/ PE(20:2/20:3)				
PC(40:4)	1.00	2.42	2.07	1.76
PE(18:0/22:4)				
PC(16:0/20:4)	1.00	1.50	0.88	0.87
PE(18:1/18:3)/ PE(16:0/20:4)				

Choline and ethanolamine ether lipids constitute around 15-20% of total phospholipids in the membranes, fulfilling here an important structural role. A general view of our results evidences a marked increase in the intensity of choline ether lipids in melanoma cell lines. Conversely, a reduction in ethanolamine ether lipids is observed in the malignant cell lines, what correlates with the results published in other works in migratory breast cancer cells compared to epithelial cells[163] and metastatic colon adenocarcinoma cell lines versus normal tissue[164]. Indeed, enhanced ether lipids presence has been related to the proliferative phenotype and tumorigenic potential of cancer cells[125]. The first steps of the ether lipid biosynthesis are carried out in the peroxisomes, being AGPS (alkylglycerone phosphate synthase) responsible for the first step of the pathway. This enzyme is overexpressed in aggressive melanoma, breast and prostate cancers and its inactivation is followed by dismissed ether lipid synthesis and decreased cellular motility and invasiveness in both breast and melanoma cells[72]. The results obtained for ether lipids in our study follow the same trend as choline and ethanolamine glycerophospholipids, suggesting that there is an increased presence of choline molecule and scarce presence of ethanolamine head group in melanoma cells. A common feature observed in cancer cells is the decreased PC/Choline ether lipids ratio, which is also present in our results, as primary and metastatic melanomas show a dramatic decrease in PC/Choline ether ratio (0.3) compared to skin melanocytes (1) **(Table 19)**. However, nevus melanocytes exhibit a marked increase in PC/Choline ether lipids ratio (2.9) compared to skin melanocytes (1). These results suggest that peroxisomes might be involved in nevus and melanoma development and progression. However, not relevant differences were observed for ethanolamine lipids, in neither melanoma nor nevus cells.

Despite having a relative minor presence, anionic glycerophospholipids including phosphatidylserine, phosphatidylinositol and phosphatidylglycerols are mainly present in the cytoplasmic face of the bilayers and have an important structural role[158]. Indeed, they participate in determining the surface charge of the membranes, and hence modulate interactions with the positively charged regions of peripheral and membrane proteins[156]. Phosphatidylserine in particular, interacts with strikingly important signaling molecules in cancer cells. For instance, PS binds to Ras and Rho family GTPases, as well as tyrosine kinase Src, modulating their mobilization to the membrane and their subsequent activation[57]. Moreover, PS binds to Akt, what activates Akt signaling pathway, promoting cell survival[165]. The biosynthesis of PS in mammal cells is carried out by a base-exchange reaction, as the head-group of an existing PC or PE is replaced by L-serine. This reaction is catalyzed by PSS1 (phosphatidylserine synthase 1) if the exchange is made from PC, whereas PSS2 acts for the replacement of ethanolamine[57]. Our results suggest that PSS1 might be inhibited in primary and metastatic melanoma cells, as the conversion from PC to PS with the same acyl-chains seems to be decreased in malignant cells **(table 36)**. However, no significant variation has been observed for the conversion of PE to PS, therefore, no changes in PSS2 activity are expected. Moreover, an important intracellular function of PS is the synthesis of PE via the mitochondrial PS decarboxylase enzyme (PSD), which is found overexpressed in zebrafish melanoma model[132]. Indeed, in our results we can see a clear increase in PE in one if the three PE and PS molecules that share the same acyl-chains and a very slight increase in the other two molecules **(table 37)**. In summary, our results contribute to explain the accumulation of PC in malignant cells as the conversion of PC to PS via PSS1 seems to be downregulated. In addition, since there are low levels of PE in our cancer cells, the conversion of PE to PS via PSS2 might be inhibited, and the decarboxylation of PS to produce PE is slightly increased in melanoma, although few molecules appear to be involved, so further research is needed to make any significant conclusion.

Table 36. PC/PS ratio of the intensities obtained for the different PC and PS species that share the same acyl-chains, related to control group (skin melanocytes=1)

	PC/PS ratio related to skin melanocytes			
	Skin melanocytes	Nevus melanocytes	Primary melanoma	Metastatic melanoma
PC(16:0/18:1)	1.00	0.98	1.50	1.63
PS(16:0/18:1)				
PC(18:1/18:1)/PC(18:0/18:2)	1.00	1.54	1.82	2.57
PS(18:2/18:0)/PS(18:0/18:2)/PS(18:1/18:1)				
PC(18:0/18:1)	1.00	2.04	2.21	3.98
PS(18:1/18:0)/PS(18:0/18:1)/PS(16:0/20:1)				
PC(18:0/20:2)	1.00	1.04	3.89	4.63
PS(18:0/20:2)/PS(18:1/20:1)				
PC(40:2)	1.00	0.90	1.61	2.23
PS(18:1/22:1)/PS(18:0/22:2)				

Table 37. PE/PS ratio of the intensities obtained for the different PE and PS species that share the same acyl-chains, related to control group (skin melanocytes=1).

	PE/PS ratio related to skin melanocytes			
	Skin melanocytes	Nevus melanocytes	Primary melanoma	Metastatic melanoma
PE(34:1) / PS(16:0/18:1)	1.00	0.74	1.05	0.99
PE(18:0/18:1) / PS(18:1/18:0)/PS(18:0/18:1)/ PS(16:0/20:1)	1.00	0.83	1.76	1.89
PE(18:1/20:1)/PE(18:0/20:2) / PS(18:0/20:2)/PS(18:1/20:1)	1.00	0.81	1.19	1.07

Other anionic glycerophospholipid subclass present in cell membranes, phosphatidylinositol, has increased intensity in the metastatic cell lines in our study. This same trait had been detected in metastatic melanoma by other authors[166]. In the PI-cycle, there is a reversible phosphorylation of PIs driven by PI3K, which has been found mutated in many cancers, including melanoma. The product of this enzyme, PIP_3, is a potent pro-tumorigenic second messenger that has been strongly related to metastatic capacity and it modulates cell proliferation, growth and migration[166]. However, the lipidomic analyzes that were used in our work hamper the detection of phosphorylated PIs, so we can only confirm the increased levels of PIs.

The last anionic lipid subclass found in the lipid content of the membranes are phosphatidylglycerols, although they are found in very low amounts (1-2% of the total glycerophospholipids)[65]. Our results show that there is no difference in PG levels in skin melanocytes (1.00) and metastatic melanomas (1.02) cell lines. However, decreased levels were found for nevus melanocytes (0.65) and primary melanomas (0.54). PGs are precursors of cardiolipin molecules, an important lipid for maintaining proper mitochondrial function. However, we did not detect cardiolipins in our work, as their molecular mass is greater than 1,500 units, and with the mass spectrometry strategy used we could only detect lipid molecular species with masses between 50 and 1,200 units.

Together with glycerophospholipids, sphingolipids constitute another major lipid class present in cell membranes. Unlike GPLs, sphingolipids fundamentally contain saturated or trans-unsaturated acyl side-chains, forming a taller, narrower cylinder structure, thus, augmenting the packing density in the membrane and decreasing the mobility within the bilayer. The rigid structure of ceramides also participates in reducing the fluidity of the bilayers[156,157]. Sphingomyelin is the most abundant sphingolipid subclass in mammal bilayers[158], and has a choline head group within its structure. In our results, we have found a marked drop in the intensity of sphingomyelins and ceramides in malignant cells. Therefore, we suggest that this contributes to increased fluidity in the bilayers of cancer cells, and goes in accordance with the results obtained in increased choline lipids and choline/ethanolamine lipids ratio. Indeed, this feature promotes the mobility and metastatic capacity of malignant cells, since they have increased deformability capacity[145,161,167]. Edmond et al proposed that epithelial-to-mesenchymal transitions modify sphingolipid levels to increase membrane fluidity and therefore facilitate cell migration[168].

The low levels of sphingomyelin species could be partially explained due to the reduced synthesis of SMs. Indeed, sphingomyelin synthase 1 (SMS1) has been found downregulated in several cancer tissues. In melanoma concretely, the levels of SMS1 have been proven to be

decreased in both primary and metastatic melanoma compared to skin and nevus melanocytes. Moreover, this hallmark has been linked to worse patient outcome in metastatic melanoma and increased migration in melanoma cells, suggesting that low levels of SM participate in melanoma progression[166,169]. In addition to decreased synthesis, increased SM metabolism might also participate in the low levels of SM that we have found in malignant cells. After the catabolism of SM, a ceramide is formed. We have detected that the ratio of sphingomyelin/ceramide species that share the same acyl chains is decreased in melanoma, this is, the intensity of ceramides is increased compared to their corresponding sphingomyelin, suggesting that this conversion is upregulated in our melanoma cell lines **(Table 38)**. Although some authors have determined that the ceramide forming enzymes, sphingomyelinases, are downregulated in melanoma cells[170–172], and thus, this conversion is decreased, the results obtained by other authors in clear cell renal cell carcinoma[155] go in accordance with our results. Moreover, upon the activity of sphingomyelinase, a phosphocholine is also released, which could be used for the increased synthesis of choline lipids, and thus, go in line with our previous results.

Table 38. SM/Cer ratio of the intensities obtained for the different SM and Cer species that share the same acyl-chains, related to control group (skin melanocytes=1).

	SM/Cer ratio related to skin melanocytes			
	Skin melanocytes	Nevus melanocytes	Primary melanoma	Metastatic melanoma
SM(d18:1/16:0)	1.00	1.44	0.46	0.53
Cer(d18:1/16:0)				
SM(d18:1/18:0)	1.00	0.79	0.48	0.36
Cer(d18:1/18:0)				
SM(d16:1/24:1)/SM(d18:1/22:1)/ SM(d18:2/22:0)	1.00	0.93	0.39	0.44
Cer(d18:2/22:0)				
SM(d18:1/22:0)	1.00	1.12	0.35	0.39
Cer(d18:1/22:0)/Cer(d16:1/24:0)				
SM(d18:1/24:1)	1.00	0.95	0.36	0.36
Cer(d18:1/24:1)				
SM(d18:1/24:0)	1.00	1.67	0.51	0.55
Cer(d18:1/24:0)				

Together with phosphocholine, ceramide is also yielded after the metabolism of sphingomyelin. Ceramides are very important bioactive lipids, and, in our study, melanoma cells have shown markedly reduced levels of ceramides. Uchida et al described that the exogenous addition of ceramides to melanocytes cell cultures blocked melanocyte proliferation after the inactivation of Akt, for instance[173]. Ceramides are considered non-tumorigenic molecules, as high levels of ceramides are associated with growth arrest, senescence, apoptosis and autophagy[174,175]. Moreover, ceramides decrease the metastatic potential of tumor cells as they regulate the surface expression of integrins[175]. Indeed, some chemotherapeutic treatments including daunorubicin, camptothecin, etoposide, rise the ceramide levels within the cells leading to apoptosis[84,176]. Tumor cells however, increase the metabolism of ceramide into other sphingolipids with pro-survival roles. For instance, acid ceramidase (ACDase) is known to be overexpressed in melanoma and other tumor cells and facilitates the conversion of ceramides into sphingosine-1-phosphate, which is upregulated in melanoma cell lines compared to normal melanocytes and promotes tumor cell proliferation and migration[174,175]. Moreover, *in vitro*

studies with dacarbazine have shown a dose- and time-dependent decrease in ACDase levels in therapy-responding cell lines, as it degrades ACDase in lysosomes and increases the intracellular levels of 16-and 18-carbon ceramides[174,175,177]. Interestingly, pharmacological inhibitors of ACDase increase ceramide levels, what synergistically improves the cytotoxic effects of chemotherapeutic agents and radiotherapy on melanoma cultures[174,176]. Moreover, high expression of ACDase has been correlated with resistance to melanoma treatment[84,174,176]. In reference to our results, primary melanomas have shown lower ceramide levels than metastatic melanomas, what goes in line with the findings of Realini et al and Leclerc et al. In their work, they found that proliferative melanomas showed higher ACDase levels and activity than invasive cell lines[174,178].

The low levels of ceramides in melanoma could be not only due to their increased catabolism, but also because of reduced *de novo* synthesis by ceramide synthase enzymes (CerS)[175]. Six different isoforms of this enzyme have been identified, and each of them produces different chain length-ceramides that perform distinct roles in the tissues. For instance, CerS1 generates C18-ceramides, which perform pro-apoptotic functions and has been found repressed in head and neck cancer cells[84,179], thus, reducing the levels of these ceramides. In addition, CerS6 produces C16-ceramides and is downregulated in melanoma and other cancer tissues[84,175,179]. Moreover, the silencing of CerS6 in melanoma cells was traduced into enhanced proliferation and invasion of these cells, among other alterations[180]. Our results suggest that both CerS1 and CerS6 might also be downregulated in our melanoma cells, as the levels of short-chain (C16 and C18) ceramides are decreased in malignant cells compared to skin melanocytes. However, long-chain (C24) ceramides have augmented their levels upon nevus and melanoma development. Long-chain ceramides are synthetized by CerS2, which could be upregulated in melanoma and nevus cells according to our lipidomic results. In fact, C24 ceramides have been related with cell survival roles[175,181].

Concerning non-structural lipids such as free fatty acids, diglycerides and triglycerides, our study shows dismissed intensity for these lipids in malignant cells, especially in the triglycerides content in metastatic melanoma. However, increased levels of these lipid classes have been largely described in tumor tissues, so further investigation is needed to determine the levels of these three lipid families in melanoma.

2. Finding new lipid biomarkers for melanoma

The global lipidomic results obtained in UHPLC-MS/MS confirmed that the lipid content is altered between melanocytes and melanoma cells. Indeed, the examination of the intensities obtained for each lipid species using supervised OPLS-DA analysis illustrated that there is a panel of 45 lipids, which can discriminate melanoma cells from melanocytes. These results confirmed our hypothesis, that there is an alteration in concrete lipid species between normal skin melanocytes and different stages of melanoma. Hence, we guided our research to find melanoma biomarkers employing a tool with translational potential: functional cell membrane microarrays. Here, membrane suspensions of the cell lines studied were immobilized, and MALDI-MS lipidomic approach was applied. This is a useful tool, as little quantity of each sample is needed, there is no need of using challenging lipid extraction methods, MALDI-MS is the gold standard method for biomarker discovery, and most of the relevant changes observed in the lipid extracts analyzed by UHPLC-MS/MS were membrane lipids. One of the main drawbacks of MALDI-MS is that it is a soft ionization lipidomic technique, so sometimes it cannot give a complete description of the acyl side-chains of the molecule. However, in this study, we have overcome it by using the molecule description that we achieved in the UHPLC-MS/MS strategy in the lipid molecules that were detected by both techniques. For this reason, some molecules could not be assigned properly, as they were not previously detected by UHPLC-MS/MS.

The first strategy to find lipid biomarkers was to perform a t-test ($p<0.05$) comparing the intensities of the different lipid molecules obtained for non-malignant (skin + nevus melanocytes) and malignant cells (primary + metastatic melanoma). Here, 116 lipid species showed significantly altered presence, and therefore confirmed that we could use this analytical approach to detect potential lipidomic biomarkers. Consequently, a multi-comparative t-test and post-hoc corrections ($p<0.05$) were employed confronting the four study groups among them. Our statistical analyses demonstrated that there are 122 lipid molecules that have discriminant levels between the four study groups **(Table 39)**.

The different PCA and clustering analyses that we performed showed that nevus melanocytes have a lipidome more similar to skin melanocytes than to melanoma cells. For this reason, we utilized nevus melanocytes as non-malignant cells in many of the statistical analyses. However, even though they are mainly benign tumors, they are still tumors so their lipidome is different from normal skin melanocytes. Indeed, we have found 11 lipids that are able to discriminate between skin and nevus melanocytes **(Table 39)**. Interestingly, 10 of these markers are also found in the comparison between skin melanocytes and primary or metastatic melanoma cells, suggesting that altered levels of these molecules could help in the transition from nevus to melanoma. Indeed, our results demonstrate that there are 48 and 54 lipids with a differential expression between skin or nevus melanocytes and primary melanomas, respectively. Interestingly, 33 of the 54 lipids that were found significant in the comparison of nevus vs primary melanoma cells were also significant for distinguishing skin melanocytes from primary melanomas. Hence, there are 21 lipids that exclusively alter their levels in the transition from nevus melanocytes to primary melanomas, and therefore, gain insight as melanoma development markers from nevus.

The difference between the lipid content of skin or nevus melanocytes and metastatic melanoma is also evident, since there are 82 and 81 lipid molecules that are potential biomarkers between skin or nevus melanocytes and metastatic melanoma **(Table 39)**, respectively.

One of the main challenges in melanoma research is finding progression markers for metastatic melanoma, since the patient outcome drops dramatically if the tumor is not detected early. Interestingly, there are three lipid species that increase their levels significantly from primary to metastatic melanoma: lipid 10, lipid 11 and lipid 22. Therefore, a deeper investigation of these concrete lipid species should be performed to evaluate their role in the progression of primary to metastatic melanoma.

Following the same trend as in the lipid extracts, 17 sphingomyelins showed a significant decrease in their intensity in malignant cells, confirming the loss of SM molecules in cancer cells. In addition, there were two DG molecules and one HexCer that presented increased levels in melanocytes compared to melanoma. On the other hand, 13 PI molecules have gained intensity significantly in melanoma, especially in metastatic melanoma. As mentioned previously, these are pro-tumorigenic molecules and have been strongly linked to the metastatic potential of the cancer cells. According to the other lipid subclasses, not all the lipids within the family follow the same trend. For instance, there is variability in the ether lipids that are formed in the peroxisomes, which have been proven to be altered in cancer. Among these, there are 18 PC ether lipids, 16 of them increase their levels in melanoma cells whereas two decrease their intensity. However, the six PE ether lipids that present discriminant levels are divided in two groups of 3 molecules, one with increased levels in melanoma and the other one with decreased levels. In addition, although we cannot confirm if they are PC or PE ether lipids, there are seven significant PC or PE ether lipids and six of them have increased levels in melanoma. These results and the ones achieved in the UHPLC-ESI-MS/MS section confirm that the peroxisomes play a role in melanoma development and progression, so further examination of these organelles is needed to understand their contribution.

The variability in the levels of the lipid molecules within the subclasses demonstrates that each molecule plays a unique role and that the composition of the head-group and the acyl side-chains is strikingly important to determine the function of each molecules. Thus, it is of interest to investigate individually the role of all the potential biomarkers detected in this work. Moreover, after confirming that the lipidome of melanoma cells and melanocytes is different, diverse and complementary lipidomic analyses should be performed to study the importance of essential lipids in melanoma, such as, phosphorylated PIs, glycosphingolipids and sterols.

Table 39. List of lipid molecules with significantly altered levels in the different statistical comparisons. The color of the * represents the study group where the lipid species is more abundant. Normal cells (skin and nevus melanocytes): purple; malignant cells (primary and metastatic melanoma): light brown; skin melanocytes: green; nevus melanocytes: yellow; primary melanoma: red; mestastatic melanoma: blue. Significance: *<0.05; **<0.01; ***<0.001; ****<0.0001.

Lipid class	Lipid specie	Normal vs Malignant	M vs N	M vs MP	N vs MP	M vs MM	N vs MM	MP vs MM
SM	Lipid 1	***		**	***	**	***	
	Lipid 2				*			
	Lipid 3	***		*	**		*	
	Lipid 4	**	*	***	*	***		
	Lipid 5	**		*	***	*	***	
	Lipid 6	****		*	**	*		
	Lipid 7	***		****	****	***	****	
	Lipid 8	***			**		*	
	Lipid 9				*			
	Lipid 10	****						*
	Lipid 11	***						*
	Lipid 12	**			**		***	
	Lipid 13	***		*	****		****	
	Lipid 14			*	*		*	
	Lipid 15	****			***		**	
	Lipid 16	**			****		***	
	Lipid 17	***			****		****	
HexCer	Lipid 18					*		
DG	Lipid 19	****				**	**	
	Lipid 20	***			*	***	****	
TG	Lipid 21						*	
	Lipid 22							*
PC	Lipid 23	***				*		
	Lipid 24			*		*		
	Lipid 25	***		*	*	*	****	
	Lipid 26	***		****	***	***	***	
	Lipid 27	***				**	*	
	Lipid 28		**	***		****		
	Lipid 29	**					***	
	Lipid 30					**		
	Lipid 31	***		**	**	***	**	
	Lipid 32	**						
	Lipid 33	***			**		**	
	Lipid 34	**					*	
	Lipid 35	**						
	Lipid 36	*		**		**		
	Lipid 37	**			*		**	

	Lipid 38	***			****		****	
	Lipid 39	****				***	**	
	Lipid 40	***				*		
	Lipid 41					*	**	
	Lipid 42	***						
	Lipid 43						*	
	Lipid 44	***		**	**	***	****	
	Lipid 45	****			**		**	
	Lipid 46	****						
	Lipid 47	***		*		**	**	
	Lipid 48	***		*		**		
	Lipid 49						*	
	Lipid 50	****						
	Lipid 51	****		***		**		
	Lipid 52	****						
	Lipid 53		**	**		***		
	Lipid 54		**	**		**		
	Lipid 55		***	***		**		
PC ether	Lipid 56	****						
	Lipid 57		*			*		
	Lipid 58	****		*	*	*	*	
	Lipid 59	****		*	*	**	**	
	Lipid 60	***		**	***	**	****	
	Lipid 61	***				**	**	
	Lipid 62	**		*		***	***	
	Lipid 63	***					**	
	Lipid 64					*		
	Lipid 65	***						
	Lipid 66	***		**	**	***	***	
	Lipid 67	***			*	*	***	
	Lipid 68	***				*	*	
	Lipid 69	***						
	Lipid 70	****		*		*		
	Lipid 71	***		*		*		
	Lipid 72	***	*			*		
	Lipid 73					*	*	
	Lipid 74	***				*	*	
	Lipid 75	***				*	*	
	Lipid 76	***				*	*	
PE	Lipid 77					*		
	Lipid 78	***		*		**		
	Lipid 79	***						
	Lipid 80	**		**	**	**	**	
	Lipid 81	***	*	*		****		

	Lipid 82			*		**		
	Lipid 83	***		*	*	**	**	
	Lipid 84	***			*			
	Lipid 85	***		**	*	*	*	
PE ether	Lipid 86	**	*			**	*	
	Lipid 87		*					
	Lipid 88	**			****	*	****	
	Lipid 89	**						
	Lipid 90	****					**	
	Lipid 91	***			**			
	Lipid 92				**			
PC/PE	Lipid 93	****						
	Lipid 94	****					*	
	Lipid 95					***		
	Lipid 96	***		**	*	**	*	
	Lipid 97					*		
	Lipid 98	****				**	*	
	Lipid 99	****				**	***	
	Lipid 100	**		*	*	***	***	
	Lipid 101	****						
	Lipid 102	****						
	Lipid 103	****				***	**	
PC/PE ether	Lipid 104	***					*	
	Lipid 105	***					**	
	Lipid 106	****						
	Lipid 107	***		**	**	**	***	
	Lipid 108	****						
	Lipid 109	****		*	*	*	*	
	Lipid 110	***				**	**	
	Lipid 111			**		**		
	Lipid 112	****						
	Lipid 113	***						
	Lipid 114	****			***		**	
PS	Lipid 115					***		
	Lipid 116	***						
	Lipid 117	***		**	**	*	**	
	Lipid 118	****		**		****	*	
	Lipid 119	****				**	*	
PG	Lipid 120	**	*	**	*	***	**	
	Lipid 121	****			**			
	Lipid 122	***						
	Lipid 123	***			*		*	
	Lipid 124	****			*		*	
	Lipid 125					***		

	Lipid 126	***						
	Lipid 127	***				**	*	
PI	Lipid 128	***						
	Lipid 129	**						
	Lipid 130	***		*	*	**	**	
	Lipid 131	***		*	*	**	**	
	Lipid 132	****		*	*	*	*	
	Lipid 133	***		**	**	**	*	
	Lipid 134	***			*		****	
	Lipid 135	**			**		*	
	Lipid 136	****		*	*	****	***	
	Lipid 137	***				**	**	
	Lipid 138	****				*	**	
	Lipid 139	***		**	**	****	**	
	Lipid 140	***				****	****	
	Lipid 141	**				****	****	
	Lipid 142	****			*	*	**	
	Lipid 143	****						

3. Phospholipase D2 promotes tumorigenic and metastatic activities in melanoma cells

Besides the structural role that phospholipids play in cell membranes, they can also be metabolized and transformed into second messengers. Phospholipases are the enzymes that hydrolyze the ester or phosphodiester bonds of phospholipids. In addition to the structural role that these enzymes play by influencing the synthesis of the bilayers, degradation and the biogenesis of organelles, they also participate in producing bioactive molecules[182]. The catabolic reactions of PCs, PEs, PIs, PGs and PSs are conducted by different phospholipases, including phospholipase A, C and D. Although each phospholipase preferentially hydrolyzes one type of GPL and regulates specific signaling pathways, they all influence cell fate by regulating several cell functions including proliferation, survival, migration, vesicle trafficking, inflammation, tumorigenesis and metastasis[141]. As a matter of fact, upregulated expression and activity of these enzymes has been found in numerous cancers. Therefore, the altered levels of different GPL subclasses that we found in the studied melanoma cell lines developed the hypothesis that different phospholipases could present altered activity and expression in melanoma, as these GPLs are substrates of the phospholipases. The study of the expression levels of PLA_2, PLC, PLD1 and PLD2 carried out by western blot in our cell lines demonstrated that PLD enzymes, especially PLD2, present increased levels from skin melanocytes to metastatic melanoma. Moreover, the upregulated expression of PLD2 in melanoma cells was confirmed by immunofluorescence. Gomez-Cambronero et al indicated that not only the expression of PLD enzymes is enhanced in cancer tissues, but also their enzymatic activity[138]. This trait was also verified in our melanoma cells, as we demonstrated that PLD activity increases significantly in both primary and metastatic cell lines.

PLD enzymes in particular, hydrolyze the PC molecules of cell membranes and transform them into mitogenic and survival signals. In particular, PLDs have been related to increased cancer cell migration and metastasis, as they participate in cancer cell progression, cytoskeleton dynamics, membrane remodeling and cell proliferation[137], for instance. After the PC breakdown by PLDs, a free choline and a PA molecule are released. As mentioned before, PCs were found clearly upregulated in the studied melanoma cells, and the production of free choline by PLDs could be used to generate more PC molecules with the specific acyl-chains that better support the carcinogenic process. Furthermore, the PA molecules produced in the process are strikingly important lipid second messenger. As it is known, PA interacts with different proteins, such as Akt and mTOR producing mitogenic signals[133,183]. In addition, PA can also be dephosphorylated to yield DAG, or hydrolyzed producing Lyso-PA; both of them are potent signaling lipids and favor the carcinogenic process[184]. Interestingly, we found elevated PA levels in the in the previously described lipidomic approaches. However, this lipid subclass was not included in the statistical analysis since its origin could not be confirmed, as it could be detected after the fragmentation of a precursor lipid[185].

PLD enzymes require PIP_2 cofactor for their activity, and we have demonstrated that PI molecules have increased presence in melanoma cells, probably contributing to augmenting the levels of phosphorylated-PI species. In fact, Epand et al demonstrated that the PI cycle increases PLD activity[61]. Another link between our previous results and increased PLD activity is that Diaz et al confirmed that sphingomyelinases potently increase PLD activity[186]. Our results in decreased SM/Cer ratio in melanoma cells suggest that SMases could present increased activity in melanoma cells, thus, we propose that this trait could also support the increased PLD activity in malignant cells. Moreover, sphingosine-1-phosphate (S1P) has also been proposed as a PLD activator[136]. Our UHPLC-ESI-MS/MS results indicate that there are low ceramide levels in

melanoma cells, which could be in part explained because of the increased conversion of ceramides into pro-tumorigenic molecules such as S1P.

The expression and activity of PLD1 and PLD2 are upregulated in several cancers, including melanoma[139]. Some authors suggest that PLD1 influences more the tumor microenvironment, while PLD2 affects preferentially tumor cells in melanoma, breast and lung cancers[145], among others. Hence, based on the western blot, immunofluorescence and enzymatic activity results obtained, we proceeded to overexpress and silence PLD2 enzyme in primary and metastatic melanoma cells to study the influence of this enzyme in basic tumorigenic processes such as cell proliferation, migration and invasion. Our results show that the augmented activity and expression of this enzyme significantly increased these processes, while the downregulation of PLD2 reduces them. These results go in line with the contributions of other authors. In lymphoma[146] and breast cancer cells[139] PLD2 has been proven to support the proliferative and invasive phenotype of the cells. Indeed, PLD2 promoted the metastatic process by phosphorylating FAK and activating mTOR and Akt[146]. It is important to highlight that the variability observed between the results obtained in PLD activity and functional analyses might be because lipase activity is not the only mechanism of action of PLD2. In fact, unlike PLD1, GEF activity is a unique property of PLD2, which is strikingly important for cell motility among other functions[135,137]. The contribution of PLD2 to cancer can be summarized in increased survival signals, enhanced proliferation, MAPK, Akt and mTOR signaling activation, apoptosis resistance, angiogenesis, evasion of growth suppression, cell invasion and metastasis, and deregulated cellular energetics[137].

CONCLUSIONS

1. Nevus and skin melanocytes have a differential global lipid fingerprint compared to primary and metastatic melanoma cells, suggesting that there is a rewiring of lipid metabolism during the development of melanoma.
2. The most relevant lipid changes detected are related to the decrease in sphingomyelin, triglycerides and free fatty acid species in primary and metastatic melanoma, together with the increase of PI species, especially in metastatic cell lines.
3. Using microarray technology we were able to detect 116 lipid species that present significantly altered levels in the comparison of normal and tumor cells, and these are potential melanoma biomarker candidates.
4. The application of microarray technology enabled the identification of three lipid species that significantly increase their levels in metastatic melanomas compared to primary melanomas. These are potential prognostic biomarkers.
5. The new functional membrane microarray technology employed is a translational biotechnological tool suitable for lipidomic studies, which could be expanded to analyze other tissues and pathologies.
6. PLD2 enzyme presents enhanced expression and activity in melanoma cells, especially in metastatic melanoma. PLD2 promotes their proliferation, migration and invasiveness thus contributing to malignancy.

APPENDIX

Table 40. List of the detected lipid species by UHPLC-ESI-MS/MS approach, the media and standard deviation of their intensity, and the % of presence of each specie within its subclass. M = skin melanocytes; N = nevus melanocytes; MP = primary melanomas; MMGL = lymph node metastatic melanoma; MMNS = subcutaneous metastatic melanoma.

	M			N			MP			MMGL		
Lipid specie	Media	SD	%	Media	SD	%	Media	SD	%	Media	SD	%
[PC(14:0/16:1)+H]+/ [PC(16:1/14:0)+H]+	48113	17531	0.70	64706	31502	0.80	126578	119731	0.64	46344	10891	0.57
[PC(16:0/14:0)+H]+/ [PC(14:0/16:0)+H]+	345673	95799	5.04	68514	76120	3.18	41854	36932	5.77	419601	10569	5.13
[PC(17:0/14:1)+H]+	51595	12681	0.75	72627	22157	1.05	39936	14361	0.65	51704	17340	0.63
[PC(16:1/16:1)+H]+/ [PC(14:0/18:2)+H]+	93745	62981	1.37	1639038	813614	1.60	1245692	344969	0.66	41446	253058	0.51
[PC(16:0/16:1)+H]+/ [PC(14:0/18:1)+H]+	930912	363129	13.58	97570	92726	10.73	64221	44258	14.80	1173303	10572	14.35
[PC(16:0/16:0)+H]+	440802	232560	6.43	56063	17052	5.23	29384	8758	6.54	323692	9000	3.96
[PC(16:0/18:2)+H]+	916724	543881	13.37	655333	300621	10.91	368452	172483	7.57	614874	189275	7.52
[PC(16:0/18:1)+H]+	1903003	155610	27.75	2297693	887253	31.34	1900550	428420	38.38	3015580	405358	36.89
[PC(16:0/18:0)+H]+	29937	17395	0.44	56106	53147	0.79	44355	69575	0.58	41241	72490	0.50
[PC(35:1)+H]+	15256	706	0.22	67426	66430	0.46	16441	23519	0.84	55272	9569	0.68
[PC(16:0/20:4)+H]+	35520	12667	0.52	59592	55553	1.06	4744	3884	0.51	35756	5364	0.44
[PC(16:0/20:3)+H]+	249705	124069	3.64	23935	16258	3.02	3610	1049	1.35	128800	4314	1.58
[PC(18:1/18:1)+H]+/ [PC(18:0/18:2)+H]+	1307992	359697	19.07	22832	14033	15.20	13385	4674	13.83	1384473	7788	16.93
[PC(18:0/18:1)+H]+	329151	139359	4.80	61091	29627	10.34	55187	28120	5.55	679062	7554	8.31
[PC(16:0/22:5)+H]	45115	16124	0.66	917	316	1.51	3507	3604	0.96	42264	3328	0.52
[PC(38:3)+H]+	53515	38748	0.78	8991	7652	1.56	5998	4190	0.24	24644	3462	0.30
[PC(18:0/20:2)+H]+	28938	1497	0.42	5858	2162	0.39	5882	1850	0.54	55550	1431	0.68
[PC(38:1)+H]+	6174	8344	0.09	6450	2848	0.07	12345	6840	0.07	6345	6234	0.08
[PC(40:6)+H]+	5899	859	0.09	10022	2673	0.15	9998	2991	0.26	14515	2509	0.18

[PC(40:5)+H]+	6964	2384	0.10	4602	2900	0.24	76702	90697	0.16	10693	4582	0.13
[PC(40:4)+H]+	9762	7504	0.14	7758	4978	0.33	6781	3483	0.07	5593	2085	0.07
[PC(40:2)+H]+	2875	1842	0.04	3447	695	0.05	3007	1338	0.04	4492	1088	0.05
TOTAL PC	**6857370**	**2215366**		**52788**	**9710**		**36696**	**13567**		**8175242**	**14965**	
[PC(O30:0)+H]+	8535	1126	4.06	3714	1494	5.28	2778	717	4.15	29084	1968	3.53
[PC(O32:1)+H]+/ [PC(P32:0)+H]+	29515	729	14.03	1561	933	17.36	2248	1410	11.02	84696	1818	10.27
[PC(O16:0/16:0)+H]+	31087	12092	14.78	8504	6022	13.96	9674	8832	19.44	122808	3901	14.90
[PC(016:0/18:1)+H]+	121025	38162	57.54	2265	1068	54.01	23214	29302	60.19	511355	6265	62.03
[PC(O36:2)+H]+/ [PC(P36:1)+H]+	20165	5906	9.59	32142	21949	9.39	67564	58067	5.20	76486	81934	9.28
TOTAL PC(O/P)	**210327**	**55763**		**234809**	**77509**		**41318**	**20508**		**824430**	**17296**	
[CE(14:1)+NH4]+	1682	204	14.19	4635	3068	6.71	3761	2120	13.57	1254	3452	16.17
[CE(15:0)+NH4]+	3692	1746	31.15	4192	3316	44.43	29819	36317	23.77	2502	30159	32.24
[CE(16:2)+NH4]+	2406	653	20.30	4640	2596	11.70	4576	1631	7.80	708	3806	9.12
[CE(17:1)+NH4]+	612	9	5.16	6483	2042	8.49	6576	2223	8.83	757	2941	9.76
[CE(18:1)+NH4]+	388	254	3.27	17838	4728	3.88	13327	5166	20.99	843	3501	10.87
[CE(20:4)+NH4]+	3071	1587	25.92	30772	6864	24.78	36326	9434	25.05	1694	5561	21.83
TOTAL CE	**11850**	**1261**		**38339**	**17674**		**26104**	**23347**		**7758**	**11718**	
[DG(32:1)+Na]+	14738	3110	9.10	145572	79531	9.32	146268	179716	7.83	8964	113277	6.99
[DG(32:0)+Na]+	7700	2197	4.75	9585	2712	5.94	4138	1055	7.31	9006	1520	7.02
[DG(34:1)+Na]+	19549	3749	12.07	34730	14393	7.04	64625	70170	16.05	20266	45047	15.80
[DG(34:0)+Na]+	10220	2294	6.31	7263	1023	10.77	13687	11870	12.55	14598	8288	11.38
[DG(36:2)+Na]+	25937	6740	16.01	11240	1669	7.83	8610	3236	8.27	9302	7011	7.25
[DG(36:1)+Na]+	5591	1608	3.45	9005	3555	3.62	14239	6303	3.76	5413	5787	4.22
[DG(36:0)+Na]+	78292	32177	48.32	5058	878	55.48	5499	2317	44.22	60719	2628	47.34
TOTAL DG	**162028**	**51875**		**39343**	**20013**		**30265**	**12877**		**128269**	**19326**	

	M			N			MP			MMGL		
Lipid specie	Media	SD	%	Media	SD	%	Media	SD	%	Media	SD	%
[SM(d18:1/14:0)+H]+	17106	34	2.18	20938	17398	1.64	16746	12902	9.23	3696	21983	1.51
[SM(d18:0/14:0)+H]+	3436	1381	0.44	6205	1489	0.23	10090	4936	1.88	756	3936	0.31
[SM(d18:1/15:0)+H]+	2044	447	0.26	11511	3290	0.47	16357	9790	1.31	2438	25115	0.99
[SM(d34:2)+H]+	5815	4961	0.74	62081	64861	1.40	33087	28859	1.89	4249	10981	1.73
[SM(d18:1/16:0)+H]+	360888	36477	46.04	10202	5874	43.71	5068	2371	27.89	92921	1807	37.92
[SM(d18:0/16:0)+H]+	53138	38811	6.78	481019	294056	4.09	813987	737887	4.29	10219	297410	4.17
[SM(d18:2/18:1)+H]+	7029	236	0.90	213371	97148	0.91	342054	200605	3.07	6698	110861	2.73
[SM(d18:1/18:0)+H]+	46049	3523	5.88	3757	1618	1.91	3720	1205	3.47	7816	1739	3.19
[SM(d18:0/20:0)+H]	1486	971	0.19	7412	5143	0.10	7277	3624	0.51	1388	2526	0.57
[SM(d16:1/24:1)+H]+/ [SM(d18:1/22:1)+H]+/ [SM(d18:2/22:0)+H]+	16206	5951	2.07	795	231	1.36	5510	4162	1.50	4260	1921	1.74
[SM(d18:1/22:0)+H]+	11923	2055	1.52	52062	10062	3.20	66342	33923	2.40	8407	25434	3.43
[SM(d16:1/24:0)+Na]+/ [SM(d18:1/22:0)+Na]+	3086	652	0.39	5832	2567	0.74	10920	3727	0.85	2337	8494	0.95
[SM(41:2)+H]+	5865	465	0.75	25108	15181	1.06	355215	425602	2.27	5432	161998	2.22
[SM(d18:1/23:0)+H]+/ [SM(16:1/25:0)+H]+/ [SM(d17:1/24:0)+H]+	1196	77	0.15	23345	13301	0.32	13452	4767	0.82	2461	8427	1.00
[SM(d18:2/24:1)+H]+	26880	24042	3.43	10681	5597	4.40	6132	2188	7.03	13748	3504	5.61
[SM(d18:2/24:1)+Na]+	3362	1141	0.43	44592	21801	0.69	12051	9991	2.91	1804	4357	0.74
[SM(d18:1/24:1)+H]+	206303	41574	26.32	25779	21505	27.28	3956	2029	23.10	60006	1568	24.49
[SM(d18:1/24:0)+H]	9303	994	1.19	1602	1119	5.96	64468	84720	5.14	15305	2985	6.25
[SM(d18:1/26:1)+H]+	2676	611	0.34	20865	4497	0.55	21823	9020	0.42	1121	3542	0.46
TOTAL SM	**783788**	**156664**		**6597**	**5035**		**2986**	**2092**		**245063**	**1842**	

[GlcCer(d18:1/22:0)+H]+/ [GalCer(d18:1/22:0)+H]+	2924	404	3.68	506455	396814	3.86	428645	363243	4.05	2084	139908	3.43
[GlcCer(d18:1/22:0)+Na]+/ [GalCer(d18:1/22:0)+Na]+	7793	378	9.80	1465555	365621	10.62	2186120	894011	8.84	6119	479424	10.06
[GlcCer(d18:1/24:1)+H]+/ [GalCer(d18:1/24:1)+H]+	13425	1369	16.88	34286	19045	14.21	31379	12934	12.97	6583	8711	10.82
[GlcCer(d18:1/24:1)+Na]+/ [GalCer(d18:1/24:1)+Na]+	38002	2724	47.78	16297	3237	37.48	18390	4904	38.36	23419	3917	38.51
[GlcCer(d18:1/24:0)+H]+/ [GalCer(d18:1/24:0)+H]+	3713	322	4.67	24979	12434	7.01	13024	7216	7.86	4960	7813	8.15
[GlcCer(d18:1/24:0)+Na]+/ [GalCer(d18:1/24:0)+Na]+	13684	2750	17.20	2074	1153	26.82	2778	2569	27.92	17655	724	29.03
TOTAL Hex-CER	**79541**	**7189**		**24645**	**12778**		**6028**	**757**		**60820**	**2456**	
[Cer(d18:1/16:0)-H]-	72329	15091	67.61	39671	15860	56.24	20421	16524	48.29	35437	13423	49.09
[Cer(d18:1/18:1)-H]-	4806	1003	4.49	2126	939	3.01	1208	454	2.86	1640	437	2.27
[Cer(d18:1/18:0)-H]-	16212	4753	15.15	5571	2955	7.90	4301	2518	10.17	7702	3551	10.67
[Cer(d18:2/22:0)-H]-	4136	550	3.87	2434	853	3.45	1675	945	3.96	2447	1107	3.39
[Cer(d18:1/22:0)-H]- / [Cer(d16:1/24:0)-H]-	2895	200	2.71	4518	1736	6.40	2809	969	6.64	5205	4156	7.21
[Cer(d18:1/24:0)-H]-	6610	2238	6.18	16222	6119	23.00	11877	4237	28.08	19750	11933	27.36
TOTAL Cer	**106987**	**17514**		**70543**	**22427**		**42292**	**22644**		**72180**	**26822**	
[LPC(18:1)+H]+	1232	162		12642	8461		34897	34999		1178	22051	
[Cer(d18:1/24:1)+Na]+	9714	1656	84.95	1259	512	65.41	4791	2134	60.24	7780	2145	60.91
[Cer(d18:1/24:0)+Na]+	1722	238	15.05	6614	3110	34.59	117039	125498	39.76	4993	38607	39.09
TOTAL Cer	**11436**	**1894**		**20697**	**7723**		**13207**	**4761**		**12773**	**7033**	

	M			N			MP			MMGL		
Lipid specie	Media	SD	%	Media	SD	%	Media	SD	%	Media	SD	%
[PE(16:0/16:1)-H]-	16882	1850	4.88	8504	6022	4.16	9674	8832	4.40	9401	3575	3.79
[PE(34:2)-H]-	53058	15081	15.32	12642	8461	6.19	34897	34999	15.86	26900	19449	10.84
[PE(34:1)-H]-	43495	6195	12.56	25994	10690	12.73	35537	24439	16.15	41702	16659	16.80
[PE(18:1/18:3)-H]- / [PE(16:0/20:4)-H]-	3947	1394	1.14	3757	1618	1.84	3720	1205	1.69	4542	1606	1.83
[PE(18:1/18:2)-H]-	17891	4055	5.17	7412	5143	3.63	7277	3624	3.31	7333	2676	2.95
[PE(18:0/18:1)-H]-	85357	18141	24.65	52062	10062	25.49	66342	33923	30.14	83788	23706	33.76
[PE(18:1/20:4)-H]-	23481	1112	6.78	16297	3237	7.98	18390	4904	8.36	21414	3529	8.63
[PE(18:1/20:3)-H]-	21382	6331	6.18	24979	12434	12.23	13024	7216	5.92	16959	7285	6.83
[PE(18:1/20:2)-H]- / [PE(18:0/20:3)-H]-	37637	18291	10.87	24645	12778	12.07	6028	757	2.74	7704	2912	3.10
[PE(18:1/20:1)-H]- / [PE(18:0/20:2)-H]-	18691	3312	5.40	9388	3500	4.60	6201	3876	2.82	8286	3637	3.34
[PE(18:1/22:4)-H]- / [PE(20:2/20:3)-H]-	13253	2966	3.83	11030	3642	5.40	16791	4165	7.63	16528	2349	6.66
[PE(18:0/22:4)-H]-	11161	2724	3.22	7549	5462	3.70	2201	475	1.00	3624	1419	1.46
TOTAL PE	**346236**	**70046**		**204261**	**58724**		**220082**	**105090**		**248179**	**66605**	
[PE(P-16:0/16:1)-H]-	5760	2541	1.68	2594	1834	1.14	10773	12981	5.50	7868	7301	2.84
[PE(P-16:0/16:0)-H]-	2864	1085	0.84	1561	933	0.69	2248	1410	1.15	2065	1617	0.75
[PE(P-16:0/18:1)-H]-	68142	26618	19.88	32142	21949	14.17	67564	58067	34.50	77065	73250	27.86
[PE(P18:0/17:2)-H]-	823	447	0.24	619	233	0.27	985	457	0.50	1041	460	0.38
[PE(P-16:0/20:5)-H]-	1264	529	0.37	1259	512	0.56	4791	2134	2.45	5522	2559	2.00
[PE(P-16:0/20:4)-H]-	21735	10032	6.34	20697	7723	9.13	13207	4761	6.74	21800	6329	7.88
[PE(P-18:0/18:2)-H]-	54900	17572	16.01	20938	17398	9.23	16746	12902	8.55	23386	19412	8.45
[PE(P-18:0/18:1)-H]-	12810	5354	3.74	11511	3290	5.08	16357	9790	8.35	26993	23382	9.76
[PE(P-16:0/22:6)-H]-	7459	3505	2.18	5832	2567	2.57	10920	3727	5.58	23317	7570	8.43

[PE(P38:5)-H]- / [PE(O38:6)-H]-	42148	22023	12.29	23345	13301	10.29	13452	4767	6.87	22646	7625	8.19
[PE(P38:4)-H]- / [PE(O38:5)-H]-	35949	19940	10.49	44592	21801	19.66	12051	9991	6.15	16467	4038	5.95
[PE(P38:3)-H]- / [PE(O38:4)-H]-	26612	20423	7.76	25779	21505	11.37	3956	2029	2.02	6651	1689	2.40
[PE(P38:2)-H]- / [PE(O38:3)-H]-	12041	6423	3.51	6597	5035	2.91	2986	2092	1.52	4154	1773	1.50
[PE(P38:1)-H]- / [PE(O38:2)-H]-	3866	1693	1.13	2435	1530	1.07	2344	1432	1.20	4049	2194	1.46
[PE(P-18:1/22:6)-H]-	8452	3951	2.47	4635	3068	2.04	3761	2120	1.92	8362	3995	3.02
[PE(P-18:0/22:6)-H]-	4255	2471	1.24	4640	2596	2.05	4576	1631	2.34	11087	3503	4.01
[PE(P40:5)-H]-/ [PE(O40:6)-H]-	13254	6882	3.87	6483	2042	2.86	6576	2223	3.36	10301	3155	3.72
[PE(P-18:0/22:4)-H]-	20512	15276	5.98	11149	7497	4.92	2557	1497	1.31	3868	1144	1.40
TOTAL PE(O/P)	**342846**	**126693**		**226808**	**77242**		**195850**	**94703**		**276641**	**149707**	
[LPE(16:0)-H]-	2809	4313	13.76	574	166	7.82	1165	880	11.54	961	622	8.74
[LPE(18:1)-H]-	4990	7754	24.44	917	316	12.48	3507	3604	34.74	2820	2941	25.65
[LPE(18:0)-H]-	2591	1272	12.69	2519	638	34.29	3606	2101	35.72	4273	2255	38.87
[LPE(20:4)-H]-	10026	17532	49.11	3336	4841	45.41	1816	1190	17.99	2940	2914	26.74
TOTAL LPE	**20417**	**30851**		**7346**	**4917**		**10094**	**7327**		**10993**	**7584**	

	M			N			MP			MMGL		
Lipid specie	Media	SD	%	Media	SD	%	Media	SD	%	Media	SD	%
[TG(44:1)+NH4]+	5550	512	0.15	17576	8542	0.19	3653	1278	0.26	1941	4471	0.19
[TG(44:0)+NH4]+	6467	1719	0.18	450753	282536	0.31	297118	197467	0.47	3645	234709	0.36
[TG(46:2)+NH4]+	14008	798	0.39	3669	1746	0.37	3178	2690	0.32	2831	991	0.28
[TG(46:1)+NH4]+	38119	376	1.06	29505	3530	0.85	19117	8748	2.17	10786	3070	1.08
[TG(46:0)+NH4]+	17935	1818	0.50	8596	4639	0.71	19535	29277	1.92	12775	4252	1.27
[TG(47:1)+NH4]+	5889	897	0.16	7609	5884	0.37	18494	23389	0.40	3547	4494	0.35
[TG(47:0)+NH4]+	6265	2080	0.17	2810	906	0.51	3740	3075	0.54	4048	1223	0.40
[TG(48:3)+NH4]+	17049	1405	0.48	2218	1009	0.43	2754	1237	0.18	2030	804	0.20
[TG(48:2)+NH4]+	104063	267	2.90	7043	2799	1.50	6136	1996	2.27	13034	1499	1.30
[TG(48:1)+NH4]+	107726	8993	3.00	2876	1654	2.07	3909	2907	8.08	48374	2098	4.82
[TG(48:0)+NH4]+	30094	1194	0.84	13880	6047	1.36	12440	3567	3.71	32098	5460	3.20
[TG(48:0)+Na]+	4455	33	0.12	60108	47963	0.30	50289	28702	0.38	5727	13036	0.57
[TG(49:2)+NH4]	6315	391	0.18	4096	2703	0.32	4307	4138	0.37	2984	1311	0.30
[TG(49:1)+NH4]	8662	1144	0.24	19604	7882	0.50	16946	7739	1.36	6282	5682	0.63
[TG(49:0)+NH4]	4473	1541	0.12	4102	1353	0.34	6349	6845	0.64	3832	2521	0.38
[TG(50:4)+NH4]+	14096	1394	0.39	5718	6533	0.58	5762	4973	0.10	1455	1211	0.15
[TG(50:3)+NH4]+	140196	14603	3.91	23986	10772	2.65	10698	6074	1.21	11827	2166	1.18
[TG(50:2)+NH4]+	396879	20554	11.06	2051	598	8.65	3730	2691	11.27	84459	1011	8.42
[TG(50:1)+NH4]+	165543	12745	4.61	160692	76618	4.86	37338	26993	14.21	115533	18823	11.52
[TG(50:0)+NH4]+	27839	3372	0.78	22684	8704	1.38	36451	26843	3.32	32086	12462	3.20
[TG(51:3)+NH4]+	4776	157	0.13	32213	18265	0.22	7764	4191	0.15	1667	10390	0.17
[TG(51:2)+NH4]+	10167	1531	0.28	4324	3598	0.45	1929	1315	1.06	5881	767	0.59
[TG(51:1)+NH4]+	7661	542	0.21	16233	13701	0.35	23624	35546	1.20	7056	6717	0.70
[TG(52:6)+NH4]+	6066	193	0.17	22564	13997	0.40	81872	132975	0.16	3374	26165	0.34
[TG(52:5)+NH4]+	26942	1778	0.75	14941	5848	1.59	37994	42626	0.52	10938	13381	1.09

[TG(52:4)+NH4]+	122066	16179	3.40	4397	1955	4.40	4318	3648	1.12	22792	1878	2.27
[TG(52:3)+NH4]+	536272	90278	14.95	3124	742	11.23	4025	1040	5.09	62727	1272	6.26
[TG(52:2)+NH4]+	523282	28021	14.59	10404	5823	13,16	18096	18968	13.73	173423	9447	17.29
[TG(52:1)+NH4]+	109215	8713	3.04	7561	3032	3.20	11017	10235	6.27	64854	6457	6.47
[TG(52:0)+NH4]+	11976	2286	0.33	7532	2762	0.66	12620	9082	1.25	13966	3642	1.39
[TG(53:3)+NH4]+	12083	3075	0.34	3436	1478	0.43	3727	3422	0.35	3661	1119	0.37
[TG(53:2)+NH4]+	14397	808	0.40	9496	1905	0.63	12767	8598	0.84	8298	13192	0.83
[TG(54:6)+NH4]+	18018	836	0.50	26160	5378	1.18	8657	5836	0.48	8571	4370	0.85
[TG(54:5)+NH4]+	45007	4777	1.25	5243	3093	2.41	12271	16826	0.73	12850	2081	1.28
[TG(54:4)+NH4]+	180617	43021	5.03	8793	3629	5.57	2646	906	1.47	24257	2652	2.42
[TG(54:3)+NH4]+	446340	134649	12.44	3563	3356	11.98	6079	6138	5.00	82959	999	8.27
[TG(54:2)+NH4]+	153104	37324	4.27	6012	6963	4.40	1195	370	3.36	45272	425	4.51
[TG(54:1)+NH4]+	21518	9899	0.60	28080	28626	0.85	13022	13997	1.03	12644	3830	1.26
[TG(56:6)+NH4]+	12795	56	0.36	96697	72751	0.74	121561	191684	0.25	2386	47693	0.24
[TG(56:5)+NH4]+	37387	7158	1.04	54084	23537	1.74	148898	211033	0.24	2538	63606	0.25
[TG(56:4)+NH4]+	50958	13482	1.42	6065	3109	1.88	3430	2157	0.21	3277	1266	0.33
[TG(56:3)+NH4]+	46277	13201	1.29	16179	2998	1.09	37903	36598	0.46	7022	15141	0.70
[TG(56:2)+NH4]+	16725	3888	0.47	14420	2616	0.48	9766	4526	0.45	6484	4404	0.65
[TG(58:6)+NH4]+	6433	827	0.18	5937	438	0.49	7226	3013	0.14	1286	2956	0.13
[TG(58:5)+NH4]+	13467	3743	0.38	2371	897	0.87	3172	1363	0.10	1100	3803	0.11
[TG(58:4)+NH4]+	9056	2773	0.25	2333	820	0.41	1582	1100	0.07	947	629	0.09
[TG(58:3)+NH4]+	8580	1934	0.24	14978	5347	0.26	27197	19503	0.14	2096	18331	0.21
[TG(58:2)+NH4]+	5043	828	0.14	4795	1657	0.17	10333	14361	0.21	2666	2923	0.27
[TG(42:0)+NH4]+	2142	328	0.06	14157	4650	0.13	22566	17263	0.14	1136	21447	0.11
[TG(56:1)+NH4]+	3170	359	0.09	3760	1260	0.15	11385	14099	0.24	2922	2898	0.29
[TG(58:1)+NH4]+	2162	110	0.06	4444	712	0.10	2031	465	0.25	2446	1024	0.24
[TG(60:2)+NH4]+	2188	489	0.06	16940	6265	0.10	6200	1247	0.14	2017	3172	0.20
TOTAL TG	**3587513**	**462250**		**47313**	**31859**		**13399**	**3722**		**1002803**	**6368**	

	M			N			MP			MMGL		
Lipid specie	Media	SD	%	Media	SD	%	Media	SD	%	Media	SD	%
[PI(32:1)-H]-	2206	319	0.92	1864	516	1.25	2328	1849	1.40	3734	1662	1.37
[PI(16:1/18:1)-H]-	19985	5408	8.36	7561	3032	5.05	11017	10235	6.61	14173	6099	5.20
[PI(16:1/18:0)-H]-	10377	2635	4.34	9496	1905	6.34	12767	8598	7.66	21963	12430	8.06
[PI(16:0/20:4)-H]-	2512	251	1.05	2371	897	1.58	3172	1363	1.90	5607	3402	2.06
[PI(36:3)-H]-	8720	945	3.65	4825	2526	3.22	3983	1889	2.39	7863	3003	2.89
[PI(18:0/18:2)-H]- / [PI(18:2/18:0)-H]-	34819	5978	14.56	14978	5347	10.01	27197	19503	16.31	43043	16619	15.80
[PI(14:0/22:1)-H]- / [PI(16:0/20:1)-H]-	20133	7386	8.42	14157	4650	9.46	22566	17263	13.54	39601	24151	14.53
[PI(18:1/20:4)-H]-	17710	3895	7.41	9005	3555	6.01	14239	6303	8.54	22773	5748	8.36
[PI(18:0/20:4)-H]-	50388	8113	21.07	39343	20013	26.28	30265	12877	18.15	45438	17181	16.68
[PI(18:0/20:3)-H]-	44095	20186	18.44	27987	14930	18.70	16187	5242	9.71	30368	12376	11.15
[PI(18:0/20:2)-H]-	16940	5367	7.08	7822	5404	5.22	7806	3353	4.68	16569	4178	6.08
[PI(40:6)-H]-	1387	330	0.58	1713	672	1.14	4116	1779	2.47	6268	2130	2.30
[PI(40:5)-H]-	2561	576	1.07	2140	591	1.43	5302	2538	3.18	6459	2358	2.37
[PI(40:4)-H]-	2546	374	1.06	2104	738	1.41	2785	1238	1.67	3653	834	1.34
[PI(40:3)-H]-	4766	1411	1.99	4336	1605	2.90	2989	1051	1.79	4966	1207	1.82
TOTAL PI	**239144**	**41132**		**149704**	**38976**		**166719**	**70001**		**272480**	**34884**	
[PS(16:0/18:1)-H]-	11496	3731	5.88	9246	4818	6.76	8928	5924	9.02	11172	2281	9.09
[PS(18:2/18:0)-H]- / [PS(18:0/18:2)-H]- / [PS(18:1/18:1)-H]-	24228	1255	12.40	8746	3675	6.39	8162	6014	8.24	9965	3146	8.11
[PS(18:1/18:0)-H]- / [PS(18:0/18:1)-H]- / [PS(16:0/20:1)-H]-	106593	20395	54.56	78426	26367	57.31	47144	21385	47.61	55321	12346	45.04

[PS(18:0/20:4)-H]-	5169	339	2.65	3938	1127	2.88	4174	1110	4.22	3629	1047	2.95
[PS(18:0/20:2)-H]- / [PS(18:1/20:1)-H]-	9614	1633	4.92	5979	2099	4.37	2678	1237	2.70	3984	1035	3.24
[PS(38:1)-H]-	14980	1870	7.67	12226	3387	8.93	11612	4215	11.73	14652	2633	11.93
[PS(18:1/22:1)-H]- / [PS(18:0/22:2)-H]-	4032	380	2.06	3510	2047	2.57	2230	611	2.25	2826	638	2.30
[PS(18:1/22:0)-H]- / [PS(18:0/22:1)-H]-	19260	1148	9.86	14783	2902	10.80	14086	4196	14.23	21288	3154	17.33
TOTAL PS	**195372**	**23724**		**136855**	**33079**		**99014**	**37440**		**122838**	**19023**	
[PG(32:0)-H]-?	688	266	1.72	662	208	2.55	2286	989	10.62	2705	1169	6.63
[PG(18:0/16:1)-H]- / [PG(18:1/16:0)-H]-	2425	1089	6.07	2076	964	7.99	4235	1456	19.67	10263	3490	25.14
[PG(16:0/18:0)-H]- / [PG(18:0/16:0)-H]-	19005	9646	47.54	10681	5597	41.11	6132	2188	28.48	9936	3146	24.34
[PG(18:1/18:2)-H]-	4937	371	12.35	2158	1151	8.31	1074	716	4.99	1746	536	4.28
[PG(18:1/18:1)-H]- / [PG(18:0/18:2)-H]-	4175	1918	10.44	2312	1481	8.90	1736	881	8.06	4322	1877	10.59
[PG(36:1)-H]-	2097	1066	5.24	2206	1317	8.49	2174	887	10.10	5220	1539	12.79
[PG(18:1/20:2)-H]-	5109	1698	12.78	2578	1494	9.92	648	268	3.01	1425	384	3.49
[PG(18:1/22:6)-H]-	1546	288	3.87	3309	2371	12.74	3242	2035	15.06	5201	2480	12.74
TOTAL PG	**39980**	**14307**		**25981**	**11619**		**21528**	**5970**		**40818**	**9867**	

	M			N			MP			MMGL		
Lipid specie	Media	SD	%	Media	SD	%	Media	SD	%	Media	SD	%
[FA(14:0-H]-	53806	10987	0.99	64706	31502	1.21	126578	119731	3.15	53908	14426	1.61
[FA(15:0)-H]-	39715	25794	0.73	68514	76120	1.28	41854	36932	1.04	33848	11179	1.01
[FA(16:1)-H]-	153989	42167	2.84	72627	22157	1.36	39936	14361	0.99	43720	17137	1.31
[FA(16:0)-H]-	1230391	299367	22.73	1639038	813614	30.64	1245692	344969	31.03	1134235	313429	33.90
[FA(17:1)-H]-	15952	7517	0.29	16377	7240	0.31	11520	5340	0.29	9425	3058	0.28
[FA(17:0)-H]-	61573	37483	1.14	97570	92726	1.82	64221	44258	1.60	47244	13489	1.41
[FA(18:2)-H]-	100049	27558	1.85	56063	17052	1.05	29384	8758	0.73	28291	9129	0.85
[FA(18:1)-H]-	1443893	409216	26.67	655333	300621	12.25	368452	172483	9.18	326533	181840	9.76
[FA(18:0)-H]-	1867914	839067	34.51	2297693	887253	42.95	1900550	428420	47.34	1537908	450345	45.97
[FA(19:1)-H]-	6823	2814	0.13	5314	2126	0.10	5989	2922	0.15	5028	1949	0.15
[FA(19:0)-H]-	21688	13350	0.40	26934	21997	0.50	15915	12790	0.40	10577	2996	0.32
[FA(20:4)-H]-	45328	33686	0.84	67426	66430	1.26	16441	23519	0.41	7541	8717	0.23
[FA(20:3)-H]-	102899	67548	1.90	59592	55553	1.11	4744	3884	0.12	5559	4864	0.17
[FA(20:2)-H]-	47947	25620	0.89	23935	16258	0.45	3610	1049	0.09	6589	4062	0.20
[FA(20:1)-H]-	52465	21585	0.97	22832	14033	0.43	13385	4674	0.33	16495	7551	0.49
[FA(20:0)-H]-	48532	15108	0.90	61091	29627	1.14	55187	28120	1.37	32277	9041	0.96
[FA(21:0)-H]-	11136	7556	0.21	13470	11655	0.25	11779	11785	0.29	5676	1879	0.17
[FA(22:6)-H]-	4186	1962	0.08	4711	4008	0.09	4270	3927	0.11	3684	2380	0.11
[FA(22:5)-H]-	9271	6500	0.17	5806	3416	0.11	6331	7501	0.16	3796	3579	0.11
[FA(22:4)-H]-	14098	5502	0.26	11009	7036	0.21	3076	2928	0.08	2440	1333	0.07
[FA(22:3)-H]-	19900	16033	0.37	18221	22528	0.34	961	552	0.02	1334	724	0.04
[FA(22:2)-H]-	8344	3684	0.15	4410	2605	0.08	1145	475	0.03	1790	796	0.05
[FA(22:1)-H]-	10002	3167	0.18	5404	2793	0.10	4288	1978	0.11	4715	1307	0.14
[FA(22:0)-H]-	43133	20774	0.80	51642	36357	0.97	39781	25916	0.99	23107	6391	0.69
TOTAL FFA	5413034	917865		5349715	1883427		4015087	1044419		3345719	874698	

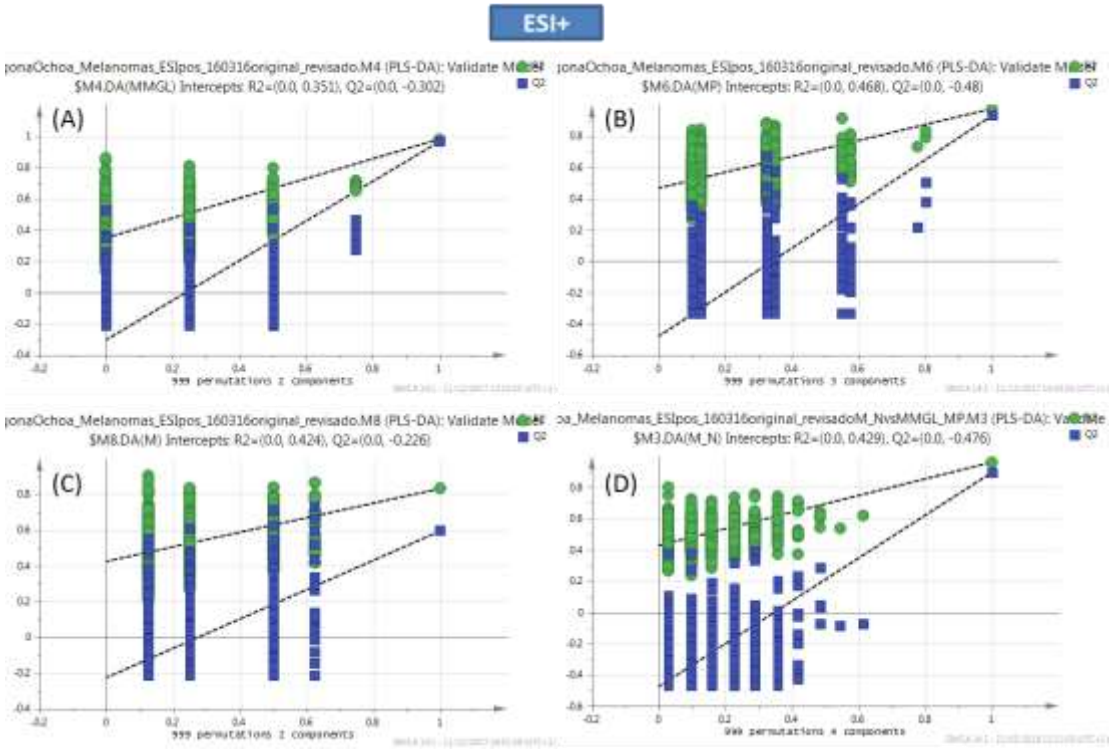


Figure 59. Permutation tests for the validation of PLS-DA analysis of the samples detected in ESI+ ionization mode classified in groups. (A) Metastatic melanoma (green) vs nevus (blue); (B) Primary melanoma (green) vs nevus (blue); (C) Melanocytes (green) vs nevus (blue); (D) Skin + Nevus melanocytes (green) vs Primary + metastatic melanoma (blue).

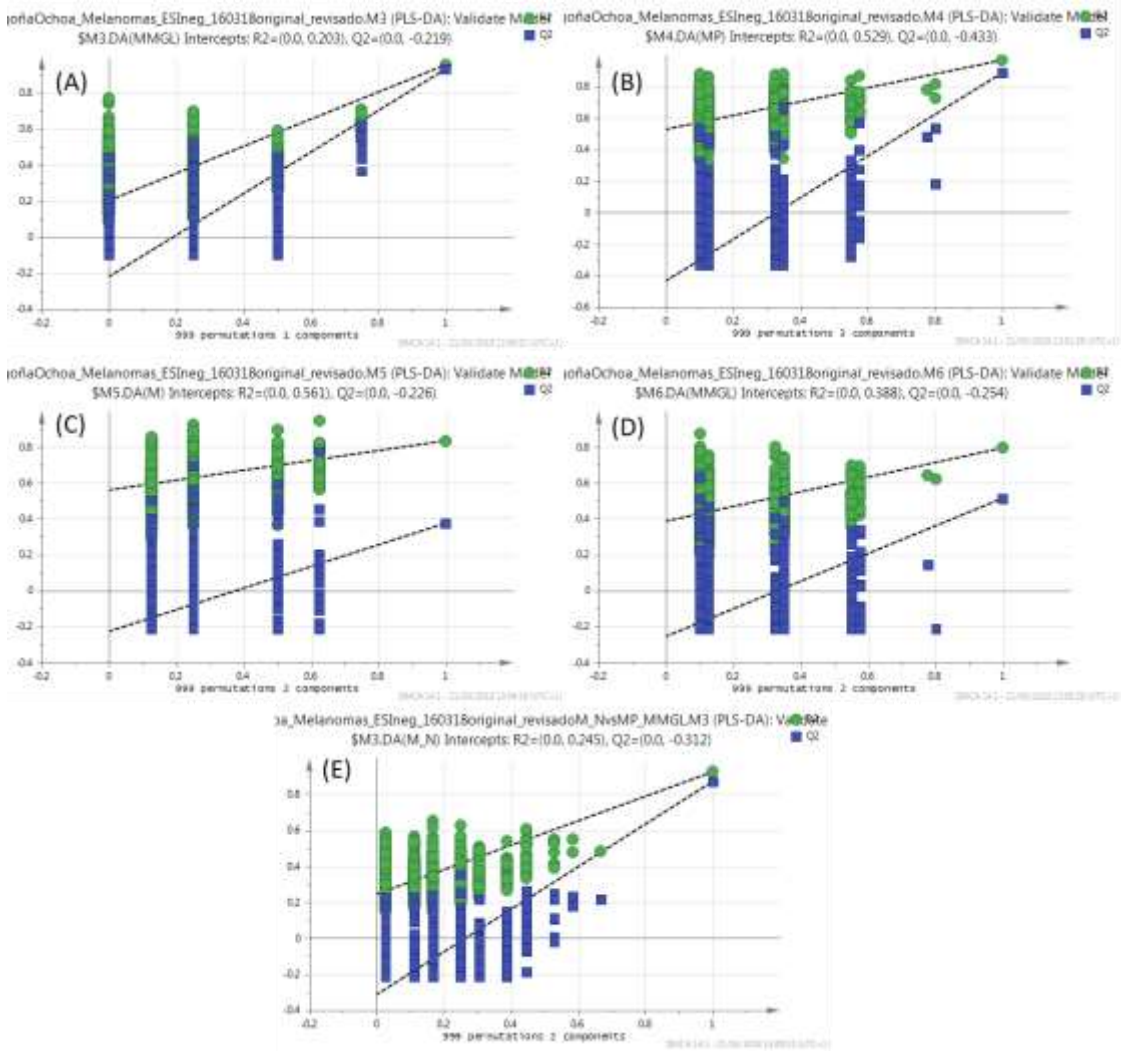


Figure 60. Permutation tests for the validation of PLS-DA analysis of the samples detected in ESI- ionization mode classified in groups(A) Metastatic melanoma (green) vs nevus (blue); (B) Primary melanoma (green) vs nevus (blue); (C) Melanocytes (green) vs nevus (blue); (D) Metastatic melanoma (green) vs Primary melanoma (blue); (E) Skin + Nevus melanocytes (green) vs Primary + metastatic melanoma (blue).

PORTADA EUSKARAZ

SARRERA

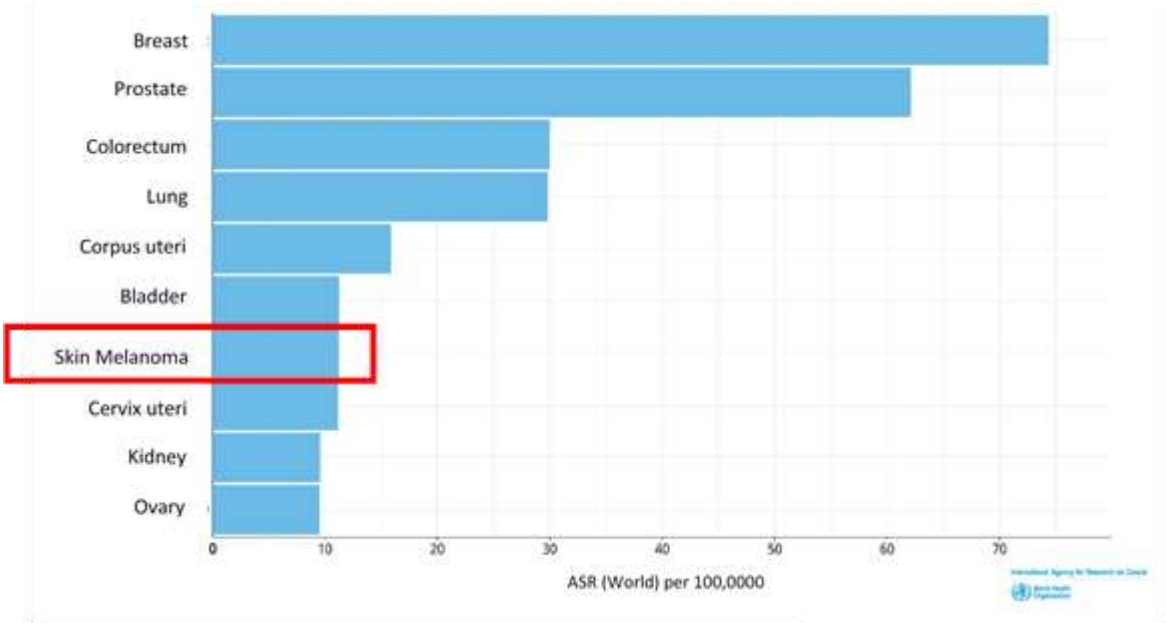
1. Melanoma

Melanoma, melanozitoak gaiztotzearen ondorioz sortzen den minbizia da. Zelula horiek kontrol gabe hazten dira tumorea sortuz. Melanozitoek melanina sortzen dute, alboko zeluletara hedatzen dutena, zelula horiek izpi ultramoreetatik babestuz. Melanoma gehienak larruazalean gertatzen dira; hala ere, gorputzeko beste leku batzuetan ere sortu daitezke, adibidez, begietan (ubeakoa) eta mukosetan. Melanoma, larruazaleko minbizi kasu guztien %4a den arren, minbizi hilgarriena da, horiek eragindako heriotzen %80aren erantzule baita. Izan ere, orduero pertsona bat hil egiten da AEB-etan melanomaren ondorioz[1].

1.1. Epidemiologia

1.1.1. Intzidentzia

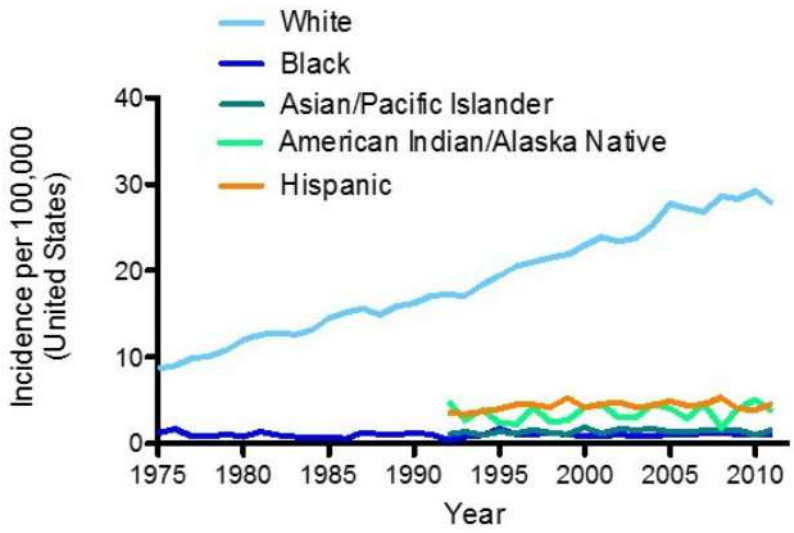
Azken hamarkadetan melanoma kasuek gora egin dute mundu mailan. Esaterako, Amerikako Estatu Batuetan, 1930ean, 1500 pertsonatik batek melanoma izateko arriskua zuen; 2011n, aldiz, arriskua handiagotu zen, eta 52 pertsonatik batek melanoma izan zezakeen[2]. Espainian, konkretuki, 2019an 150000 kasu berri egongo direla iragarri da[3]. Bestalde, Europan 2018an diagnostikatutako zazpigarren minbizi mota melanoma dela kalkulatu da, eta 100000 pertsonetatik 11,2tan diagnostikatu da **(1. Irudia)**.



Irudia 1. Europan 2018an diagnostikatutako 10 minbizi mota nagusien intzidentzia-tasa zenbatetsia. Bi sexuak eta adin guztiak kontuan hartu dira.

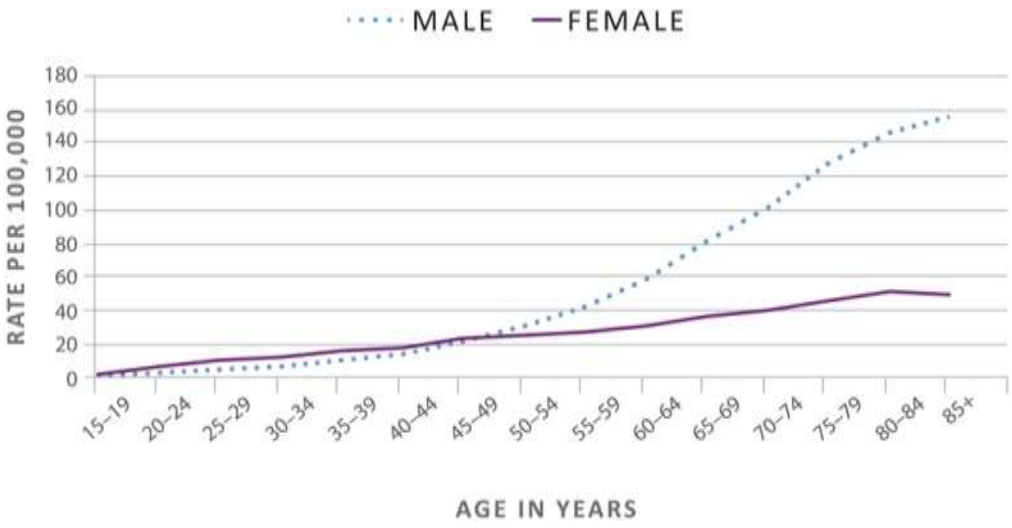
Intzidentzia etnia, sexua, adina eta ikasten den eremu geografikoaren arabera aldatzen da. Zehazki, melanomaren intzidentzia tasa dezente altuagoa da larruazal argia duten kaukasoarren artean. Jakina denez, minbizi hau sortzeko arrisku-faktore nagusiena ultramore-erradiazioa da; beraz, larruazal beltzarana duten gizabanakoen melaninak babes-hesi bat sortzen du zelulak eguzki-izpiek duten efektu kartzinogenikoaren aurka babestuz. Melanoma gehienbat kaukasoarrekin erlazionatzen den arren, afroamerikarretan, 5 urteko biziraupen-tasa baxuagoa ohi da, diagnosia beranduago egiten delako. Gainera, etniaren arabera tumoreak gorputzeko

leku ezberdinetan agertzen dira; kaukasoarretan, eguzkiarekin kontaktuan dauden eremuetan agertu ohi da, eta beltzaranetan aldiz, eguzki-izpiekin kontaktuan ez dauden lekuetan agertzen da, esate baterako, mukosetan, atzazkaletan, esku- eta oin-azpietan. Hortaz, zailagoa da eremu horietan tumoreak detektatzea[4].



Irudia 2. Melanoma intzidentzia AEB-etan, talde etniko ezberdinen arabera, 1975-2011 urteen artean[4].

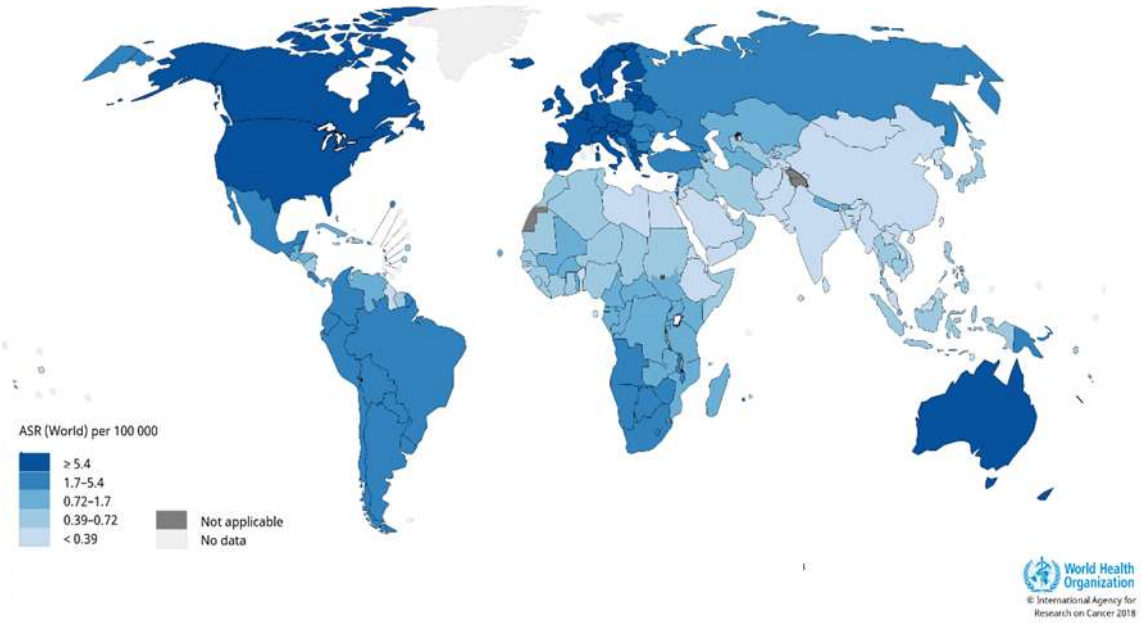
Melanoma bi sexuetan era desberdinean agertzen da. Gainera, intzidentzia-tasa pazientearen adinaren arabera aldatzen da. Tumore hori ohikoagoa da emakume nerabe eta gazteen artean, gizonezkoetan baino. 40 urtetik gora ordea, joera aldatzen da eta gizonezkoek melanoma gehiago jasaten dute, orokorrean, intzidentzia-tasa altuagoak dituztelarik. Bi sexuetan intzidentziak gora egin badu ere, ikaragarri handitu da 40 urtetik gorako emakumeen artean, ziurrenik beltzarantze-oheen erabilera jendarteratzearen ondorioz[5].



Irudia 3. Melanoma intzidentzia adina eta sexuaren arabera, AEB-tan 2007-2011 urteen artean.

Talde etniko berdineko gizabanakoen artean ere, melanoma intzidentzia kokapen geografikoaren arabera aldatzen da. Larruazal argia duten pertsonak kontuan izanda, intzidentzia tasak altuagoak dira ekuatoretik gertu dauden herrialdeetan, izpi ultramore biziagoak jasaten baitituzte. Horrela, melanoma intzidentzia-tasa altuenak Zelanda Berrian eta Australian ematen dira, bertako biztanleria gehienak larruazal argia dutelako eta herrialde horiek latitude baxuetan daudelako. Europa barruan, ordea, alderantzizko latitude gradientea sumatu da. Izan ere, erdialdeko eta iparraldeko herrialdeek, Suitzak, Herbehereak, Danimarkak, Norvegiak eta Suediak, esaterako, hegoaldeko herrialdeetan baino hiru edo sei aldiz melanoma arrisku handiagoa dute. Hori, neurri batean, biztanleriaren azaleko fototipo argiagoaren eta eguzkiaren eraginpean egoteko patroiaren ondorio izan liteke.

Iparraldeko herrialdeetako jendeak noizbehinkako eguzki esposizioa eta erredurak erraz jasaten dituzten bitartean, hegoaldeko herrialdeetako pertsonek eguzki esposizio metagarria izaten dute. Nabarmenki, Australiako eta Eskandinaviako herrialdeetako biztanleen artean iparralde-hegoalde intzidentzia-gradiente bat ikusi da, non ekuatoretik hurbilago bizi diren pertsonek melanoma izateko arrisku handiagoa duten[6].



Irudia 4. 2018-ko larruazaleko melanomaren intzidentziaren banaketa zenbatetsia mundu osoan[3].

1.1.2. Heriotza-tasa

Intzidentziaren antzera, melanomaren heriotza-tasa sexua, adina, etnia eta kokapen geografikoarekin erlazionatzen da. Haatik, heriotza tasak ez du intzidentziaren joera berdina jarraitu. Izan ere, azken 20 urteetan hilkortasuna egonkortu da, ziurrenik sentsibilizazio, diagnostiko goiztiarra eta arreta mediko eta kirurgikoen aurrerapenak direla eta[4]. Intzidentzia bezala, hilkortasuna ere altuagoa da latitude baxuetan kokatuta dauden herrialdeetan, ekuatoretik gertu. Sexu eta adinari dagokionez, gizonek heriotza-tasa altuagoak dituzte, eta hilkortasunaren gailurra 70 urtetik haratago dago. Estatu batuetako talde etnikoen artean, hilkortasun tasa altuena Kaukasoarretan ematen da. Kaukasoarrek melanoma izateko joera handiagoa badute ere, afroamerrikarretan, normalean, beranduago detektatzen da eta horregatik, biziraupena txikiagoa da talde etniko horretan. Izan ere, 5 urteko biziraupen tasa igo

egin da kaukasoarren artean azken hamarkadan, eta afrikar-amerikarretan aldiz, behera egin du. Hala ere, joera horren erantzule egoera sozioekonomikoa izan daiteke[5].

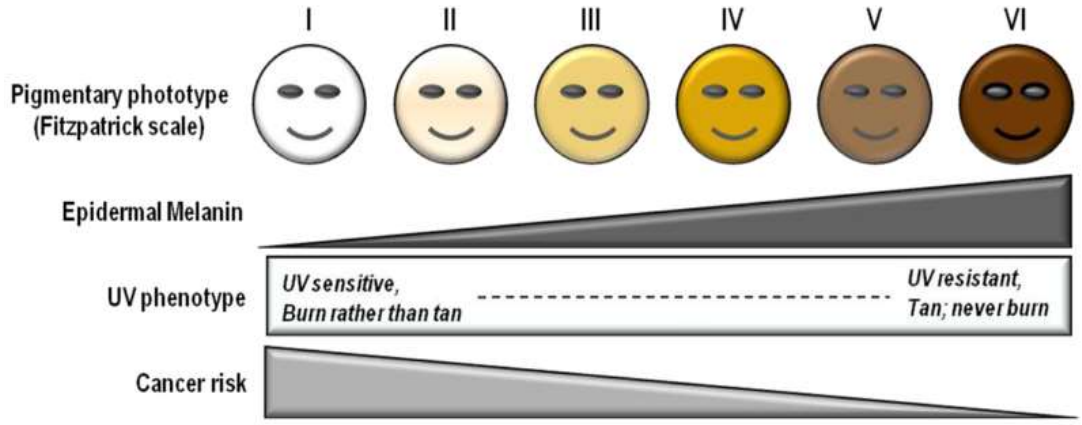
1.2. Etiologia

Melanomaren etiologia ezezaguna den arren, minbizi horren sorrera eta garapena laguntzen duten arrisku-faktore batzuk identifikatu dira. Faktore horiek bi multzotan bana daitezke: faktore intrintsekoak edo berez gaixoarenak direnak eta faktore estrintsekoak edo ingurumenekoak.

1.2.1. Faktore intrintsekoak

Lehenago adierazi den moduan, pazientearen **adina** eta **sexua** faktore garrantzitsuak dira melanoma garatzeko aukera ezartzeko. Horrela, minbizi hori agertzeko arriskua altuagoa da adinekoetan, emakume zein gizonetan. Melanoma diagnostikatzen den batez besteko adina 60 urte dira. Orokorrean, gizonezkoetan arrisku handiagoa dago non 80 urtekoetan ratioa 2 gizonezko: 1 emakumezko den; aldiz, **3. Irudian** ikusten den bezala, 40 urtetik beherakoen artean, melanoma tasa emakumezkoetan altuagoa da AEB-n[2].

Pertsona baten larruazaleko melanina kantitateak bere **azaleko fototipoa** zehazten du, eta horrek pertsona jakin batek melanoma garatzeko duen joera definitzen du. Thomas B. Fitzpatrickek 1975 urtean, Fitzpatrick eskala ezarri zuennon zenbakiekin sailkatu zituen gizabanakoak erradiakzio ultramoreekiko duen sentiberatasuna. Ondorioz, pertsona horrek melanoma garatzeko duen arriskua erlazionatzen da eguzkiarekin erretzeko duen joera eta bere melanina kopuru basalarekin. Larruazal argia, ile-hori edo ile-gorria, oreztak, eguzkiarekin erretzeko joera altua eta belzteko ezintasuna duten gizabanakoek Fitzpatrick eskalan puntuazio baxua izango lukete, eta horiek arrisku handiagoa dute melanoma garatzeko. Aitzitik, Fitzpatrick-en puntuazio altuagoa duten pertsonek larruazal ilunagoa dute, ez dira eguzkiarekin erretzen, erraz beltzaratzen dira eta hori melanoma garatzeko arrisku txikiagoarekin lotzen da[2,4].



Irudia 5. Fitzpatrick eskala. Zenbakizko sailkapena gizabanako batek erradiazioultramore eta melanomaren garapenarekiko duen sentikortasuna zehazteko. D'0razio *et al*.-tik hartuta.

Melanoma kasuen %5-10ek soilik dute jatorri genetikoa, baina melanoma aurrekari familiarrak gaixotasun hori garatzeko arrisku handiagoarekin erlazionatu izan da. Zehazki, lehen mailako senide batek melanoma izan badu, arriskua 1,7 handiagotzen da, eta gurasoetako batek

melanoma anizkoitza badu, arriskua 61,78 aldiz handiagoa izango litzateke[2,5]. Nahiz eta oraindik ez dakigun arrisku horren igoera heredatutako aurrejoera genetiko baten edo partekatutako ingurumen-faktoreen ondorio den, gaixotasun genetiko batzuk melanoma garatzearekin lotu dira. Gehien aztertu den nahasmendua Xeroderma pigmentosoa da[2,4,7]. Gaixotasun autosomiko errezesiboa da hori, eta melanoma izateko arriskua 1000 bider handitzen du. Sindrome hori duten gaixoek nukleotidoen erauzketa bidezko konponketa (NER) mekanismoa akastuna dute, zeinak DNA-ren lesioak zuzentzen dituen. Beraz, zelulek ezin dute erradiakzio ultramoreek sortutako DNA-ren kaltea konpondu, eta banakoek UV izpiekiko hipersentikortasuna garatzen dute. Tumoreen %65 eguzkiaren eraginpean dauden eremu anatomikoetan sortzen dira. Beste alterazio bat orezta atipiko aniztun melanoma familiarra (FAMMM) da. Hori, bai melanoma eta bai nevus displasikoen aurrekariak dituzten bi ahaide zuzen edo gehiago izatea bezala deskribatzen da. Bereziki, 50 urterekin, sindrome horrekin diagnostikatutako pertsona batek melanoma izateko daukan arriskua %49 handiagoa da, eta 72 urterekin, %82 handiagoa. Nahasmendu hau CDKN2A genean sortutako mutazioek eragiten dute, p16 eta p14 proteinak kodifikatzen dituztenak[2,4,5,7]. Gainera, melanoma kasuen %90ean Bcl-2 proteinaren gainadierazpena dago, eta hori apoptosiaren inhibitzaile bat da. mTOR bidea, zelula-zikloaren funtsezko erregulatzailea, kasuen %67-77an aktibatzen da. MAPK/ERK bidearen aktibazioa areagotzen duten mutazioak BRAF kinasaren aktibazio iraunkorra eragiten du melanomen %60-80an, eta melanozitoen ugaritzea errazten du[8].

Gainera, edozein motatako azaleko minbiziaren aurreko **historia pertsonalak** melanoma izateko aukera areagotzen du. Melanoma aurretik izan duten pertsonek %8ko arriskua dute bigarren melanoma bat izateko[4,7]. Bestalde, beste gaixotasun batzuk melanoma garatzeko probabilitate handiagoarekin ere lotuta daudela ikusi da. Sistema immuneak funtsezko papera jokatzen du gorputza minbiziaren aurka babesten. Beraz, HIES-a duten pertsonek melanoma garatzeko probabilitate handiagoa dute. Era berean, organoen transplantea jaso duten pertsonek melanoma izateko probabilitatea %6-an handiagotzen da transplantea helduaroan izan bada, eta haurren transplanteetan %14ko probabilitatea dago. Gainera, bularreko minbiziarekin lotura antzeman diote. Bularreko minbizia izan duten eta BRCA2 mutazioa duten pertsonek melanoma garatzeko 2,58ko arrisku erlatiboa dute. Ez-Hodgkin linfoma edo leuzemia linfozitikoaren aurrekariak dituzten pazienteen artean, melanoma izateko probabilitate handiagoa dago; izan ere, bi gaitz horien ondoren sortu daitekeen bigarren minbizi probableenetako bat melanoma da[2].

Nevi kopuru handi baten presentzia melanoma izateko probabilitate handiagoarekin lotu izan da. Nevi eta melanoma gehienek BRAF mutazioa partekatzen dute, nevusa sor dezakeenak, baina melanoma garatzeko mutazio gehigarriak behar dira. Hala ere, orezta tipikoen eraldaketa gaiztoa arraroa da, nevi displasikoen presentziak arriskua handitzen duen bitartean. Izan ere, nevus displasiko batek arriskua bi aldiz handitzen du, eta 16 eta 40 orezta tipiko izateak 1,47 aldiz handitzen da arriskua. Nabarmenki, ez da antzeman sortzetiko nevi eta melanomaren garapenaren arteko loturarik[2,4,7].

1.2.2. Faktore estrintsekoak

Erradiakzio ultramorea melanomaren agerpenarekin lotu da zalantzarik gabe. Izan ere, gaitz horretarako kartzinogenorik garrantzitsuena da, kasuen %80ren erantzulea baita[4]. Izpi ultramoreek DNA-n kaltea eragiten dute, zeluletan mutazioak sortuz. Kaltetutako DNA konpontzeko mekanismoek ezin dute DNA horren sekuentziak ordezkatu, eta melanoma sortzen da. Eguzki-esposizio patroiak ere garrantzia du. Aisialdiko edo aldizkako esposizioa, batez ere azal argiko gizabanakoetan, melanoma eta neviak izateko arriskua %65ean handitzen du. Hala

ere, eguzki-kremen erabilerak melanoma tasa murrizten duela frogatu da, bereziki melanoma inbaditzailearena[2]. Azken hamarkadetan, solariumen erabilera oso popularra bihurtu da, alabaina, jarduera sozial hori melanomaren sorrerarekin estuki lotu da. Horrela, zenbait meta-analisiek iradokitzen dute beltzarantzeko oheak maiz erabiltzeak melanoma garatzeko arriskua hirukoiztu edo laukoiztu egiten duela, eta arrisku hori %75ean igotzen da erabilera hori 35 urte bete aurretik egiten bada[7].

Ekuatoretik hurbilen dauden herrialdeak erradiazio ultramore biziagoen eraginpean daudenez, hori melanoma tasa handiagotan itzultzen da, **kokapen geografikoaren** garrantzia nabarmenduz. Lehen aipatu bezala, Australiak eta Zeelanda Berriak, ekuatoretik gertu daudenak eta azal argiko populazioa dutenak, melanoma tasa handienak dituzte. Ezaugarri garrantzitsua izan arren, kokapen geografikoa ez da erabakigarria. Adibidez, ekuatoretik gertu egon arren, Erdialdeko Amerikak melanoma tasa baxuak ditu, pertsona gehienak azal ilunekoak baitira.

Metal astunekiko esposizioaren, hainbat produktu kimikoren eta melanomaren garapenaren arteko lotura ezarri da, konposatu horiek melanozitoen DNA-n mutazioak sortzen baitituzte. Erradiazio ionizatzaileen, metal astunen, hidrokarburo poliziklikoen (petrolioa, inprimatzeko produktu kimikoak eta produktu elektronikoak), pestiziden eta polibinilo kloruroaren eraginpean egoteak melanoma izateko arriskua areagotzen du. Hala ere, horren mekanismoa aztertzen ari dira oraindik[9].

1.3. Histopatologia

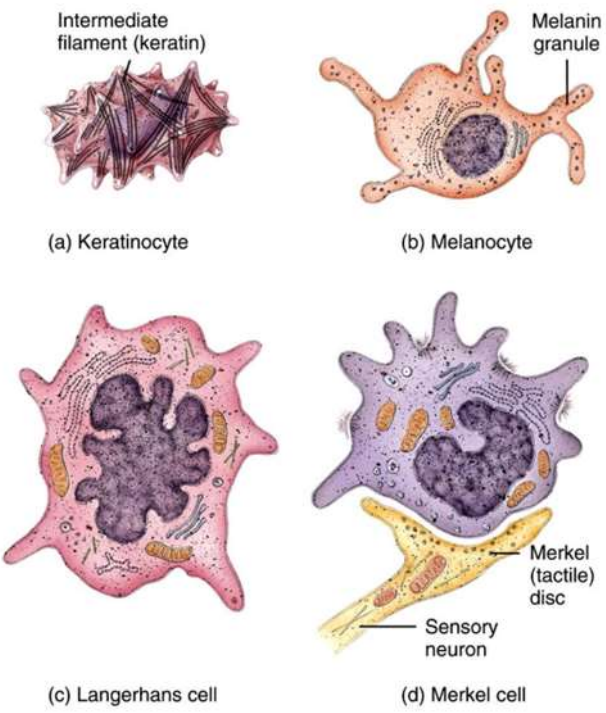
Melanozitoak gandor neuraletik eratortzen dira eta horregatik, melanoma, ubean eta mukosetan ere ager daiteke, baina kasuen %95 larruazalean sortzen dira.

1.3.1. Larruazala

Larruazala gorputzeko organorik handiena da, eta 2 m^2-ko azalera izan dezake. Beroaren, argiaren, patogenoen eta lesioen aurkako ezkutu babesle gisa jarduten du. Gainera, gorputzeko tenperatura, D bitaminaren sintesia, larruazaleko sentikortasuna (ukimena, beroa eta hotza) erregulatzen, eta uraren galera saihesten laguntzen du.

Bere egituraren arabera, bi zatitan banatzen da: epidermisa eta dermisa[10]. Bestalde, dermisaren azpian, baina larruazaletik kanpo, hipodermisa dago, larruazalpeko ehuna ere deiturikoa. Hori, nagusiki, ehun konektibo laxoz eta gantz metaketez osatzen da. Hipodermisaren eduki zelularra adipozitoek, fibroblastoek eta makrofagoek osatzen dute batez ere. Bere funtzio nagusia gantza biltzea eta gorputzeko tenperatura mantentzen laguntzea da.

Epidermisa larruazalaren kanpoko geruza da, eta gorputzaren barruko egituretarako barrera gisa jokatzen du, larruazalaren hidratazioa erregulatuz eta azalari kolorea emanez. Epitelio ezkatatsu geruzatu mehe batez osatua dago, dermisetik banatzen duen xafla basal baten gainean kokatzen dena. Egitura horretan aurkitzen diren zelula guztien artean, keratinozitoak, melanozitoak, Langerhans zelulak eta Merkel zelulak dira nagusienak **(7. Irudia)**. Zelula horien %90 keratinozitoak dira, eta keratina sortzen dute, azala babesten duen zuntzezko proteina erresistentea dena. Melanina sortzeko eta azalaren kolorea zehazteko ardura duten zelulak melanozitoak dira, epidermiseko zelulen %8 direnak. Gainera, badira Langerhans zelulak, azalean dauden mikroorganismoen aurkako erantzun immunea indartzen dutenak, eta Merkel zelulak, neurona sentikorrekin kontaktu sinaptikoa egiten dutenak eta ukimen sentsaziorako mekanohartzaile gisa jarduten direnak.



Irudia 6. Epidermisean dauden zelula mota nagusiak. (a) keratinozitoak, (b) melanozitoak, (c) Langerhans zelulak eta (d) Merkel zelulak.

Epidermisaren azpian **dermisa** dago. Ehun konektiboz osatutako geruza lodi bat da, odol-hodiak, nerbioak, guruinak eta ile-folikuluak dituena zeina epidermisera estuki lotuta dagoen xafla basalaren bitartez. Horrek, epidermisean eta dermisean dauden zelulentzako euskarri gisa, nutrizioan eta hondakinen erauzketan jarduten du.

Melanozitoak melanomaren garapenaren erantzule direnez, zelula mota horietan jarri dugu gure arreta. Ektodermo enbrionariotik sortzen dira. Beraz, hodi neurala itxi ondoren, melanoblastoak, melanozitoen aitzindariak, gandor neuraletik gorputzeko eremu ezberdinetara migratzen dira, hala nola larruazalera, ubeara, mukosetara, meningeetara, barne-belarrira eta bihotzera. Horregatik, melanoma toki horietan ere sor daiteke, nagusiki azalean garatzen den arren.

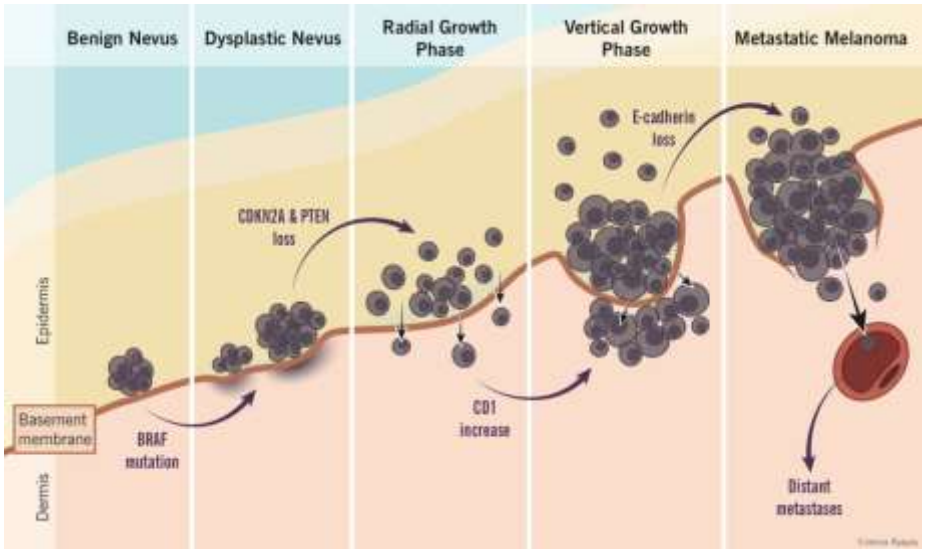
Melanozitoen dentsitatea gorputzaren eremu anatomikoaren arabera aldatzen da (handiagoa bularreko aura eta eremu genitalean). Hala ere, melanozito kopurua nahiko konstante mantentzen da etnia ezberdinetako banakoen artean. Beraz, azalaren kolorea ekoitzitako melanina kantitateak zehazten du, eta ez ordea, melanozito kopuruak.

Baldintza fisiologikoetan, melanozitoak, epidermisaren oinarrizko geruzan aurkitzen dira, horrela proiekzio dendritikoen bitartez 36 keratinozitoekin konektatuta mantentzen dira, melanina-epidermis unitatea eratuz. Melanozitoek zatikatzeko gaitasun txikia dute, zitoplasma argia, eta ez dute desmosomarik. Gainera, bere funtzio nagusia melanina ekoiztea da, larruazalerako babes gisa jarduten duen pigmentu fotobabeslea. Horrek izpi ultramoreek zeluletan eragin litzaketen ondorio kaltegarriak blokeatzen ditu, baita estres oxidatiboa eta DNA-ren mutagenesia ere. Melanozitoen barruan melanosoma deritzen organuluak daude non

melanina sortu eta metatzen den. Gero, besikula horiek melanina-epidermis unitatea osatzen duten keratinozitoei banatzen zaizkie melanozitoen proiekzio dendritikoen bidez.

1.3.2. Melanoma garapena

Lehen aipatu bezala, larruazaleko melanoma epidermisean dauden melanozitoen eraldaketa gaiztoaren ondorioz sortzen da. Zelula horiek hainbat alterazio molekular jasaten dituzte, eta horien artean, geneen mutazioak daude, zelula-zikloaren erregulazioa, zelula-bereizketa, zelula-atxikipena, zelula-seinaleztapena eta apoptosia kontrolatzen dituztenak, besteak beste[11].



Irudia 7. Clark eredua melanomaren garapena irudikatzeko[12].

Melanomaren garapena azaltzeko hainbat eredu proposatu dira. Ezagunenetako bat Clark eredua da, zeinak, **7. irudian** adierazten den bezala, melanomaren garapena azaltzen duen hainbat urratsetan oinarrituz: nevus onberak, nevus displasikoak, melanoma primarioa hazkuntza erradialeko fasean (RGP), melanoma primarioa hazkuntza bertikaleko fasean (VGP) eta melanoma metastatikoa. Progresio-eredu hori urratsez urratseko eraldaketa lineal bezala azaltzen den arren, melanoma tumore askok, ez dute modu ordenatu bat jarraitzen. Adibidez, RGP edo VGP melanomak aurretik zeuden nevusetatik edo melanozito normaletatik sor daitezke. Gainera, RGP edo VGP tumoreek zuzenean egin dezakete aurrera tumore metastatikoetara[12].

Clark ereduko lehen gertaera, nevus onbera baten agerpena da, melanozito epidermikoek, euren hazkuntzaren kontrola eraldatzen duten aldaketa molekular ezberdinak jasaten baitituzte. Hala ere, nevusetako melanozitoen proliferazioa mugatua da; zelulak onkogeneen eraginez seneszentzia fase batean sartzen dira, eta oso gutxitan egiten dute aurrera melanoma sortuz. Klinikoki, gainazal eta ertz erregularrak dituzten orezta marroi gisa deskriba daitezke. Identifikatu diren aldaketa molekular batzuk MAPK seinaleztapen bidearen aktibazio aberrantea eragiten dute, melanozitoen proliferazioa handitzea eragiten duenak. ERK-MAPK bidearen aktibazio konstitutibo hori N-RAS-en ematen diren mutazioen ondorioz gertatzen da, melanomen % 15ean ematen dena; BRAF-en mutazioa bestalde, melanoma kasuen %50ekin lotu da. BRAF mutazioen maiztasuna melanometan ikusitakoaren antzekoa da, eta, beraz, alterazio gehigarriak gertatu behar dira melanozitoak gaizto bihur daitezen. Autore batzuek iradokitzen dute BRAF mutazioek seneszentzia zelularra eragiten dutela, zelula-zikloaren inhibitzailea den

kinasa 4A-k (INK4A) adierazpena areagotzen duelako. Proteina horrek zelula-zikloa gelditzea eragiten du, eta beraz, BRAF-ek sortutako hazkuntza-zelularraren estimulazioa mugatua da[12].

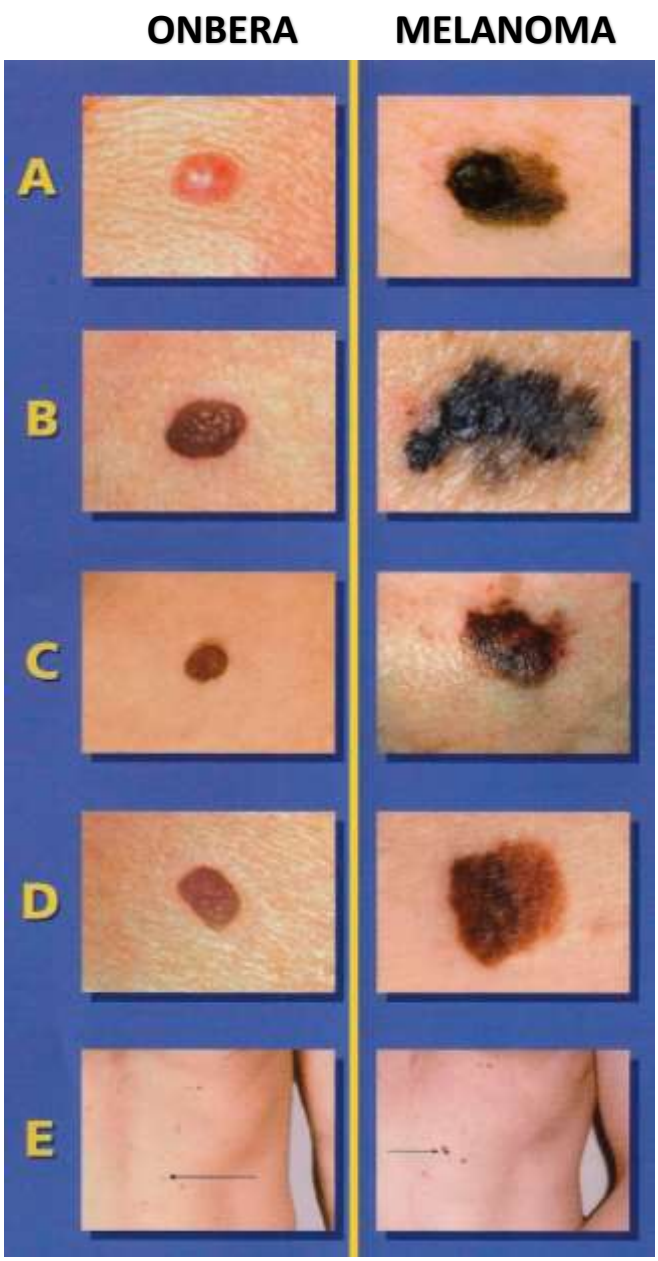
Tumore onbera horiek, nevus displasikoetan bihur daitezke, nevus onbera eta melanomaren arteko minbiziaren aurrekotzat hartzen direnak. Nevus displasiko horien aurretik zegoen tumore onbera batetik sor daitezke, edo lesio berri gisa. Nahiz eta arrisku faktoretzat hartu eta melanomaren aitzindari izateko gaitasuna izan, nevus displasiko gehienak egonkor mantentzen dira denboran zehar, eta ez dira melanomatan bilakatzen. Izan ere, melanoma kasuen %20 baino ez dira garatzen minbiziaren aurreko lesio horietatik abiatuta. Zenbait ikerketek ondorioztatu dutenez, nevus bat melanoma bihurtzeko arriskua 1-200000tan da 40 urte beherako pazienteetan eta 1-33000tan 60 urtetik gorako gizonetan[13]. Klinikoki, orezta horiek 5 mm-tik gorakoak dira, ertz irregularrekin eta koloreaniztasunak dira, eta histologikoki, zelulek atipia zitologikoa erakusten dute. Maila molekularrean, zelula-hazkuntza, DNA konpontzeko makineria eta zelula-heriotzarako suszeptibilitatea aldatzen dituzten alterazioak jasaten dituzte[12].

Azkenik, zelulek mugarik gabe hazteko gaitasuna har dezakete, tumore gaiztoak osatuz. Gainera, melanomek bi hazkuntza-fase desberdin dituzte, emaitza klinikoa zehaztuko dutenak. Lehenengo fasean tumorea alboetara hazten da epidermisean zehar eta ez du metastasirik sortzen. Horrek urteak iraun ditzake, eta tumorea kirurgia bidez kentzen da, gutxi gorabehera %100 errekuperazio-tasa lortuz. Bigarren fasea VGP da, non tumoreak sakoneran hazteko gaitasuna hartzen duen eta dermisa eta hipodermisa inbaditzen dituen, metastatiak sortuz.

Melanoma tumore mota oldarkorrenetako bat da, eta biziraupen tasak esanguratsuki jaisten dira fase metastatikoa diagnostikatzen denean. Metastasi bat osatzeko, zelulek zelulen arteko atxikipenak galtzen dituzte, tumore primariotik bereizi eta inguruko estroma inbaditzen dute. Melanoma-zelulek migrazio-mekanismo desberdinak erabiltzen dituzte barreiatzeko: hodi linfatikoen edo odol-hodien bidezko hedatze intrabaskularra, edo angiotropismo izeneko migrazio estrabaskularra. Azken hori horrela definitzen da: tumorearen hedapena, zirkulazio-sisteman sartu gabe; izan ere, zelulek odol-hodietan dauden perizitoak imitatzen dituzte, eta gainazal baskular abluminalean zehar migratzen dute, hodietan sartu gabe. Melanoma zelulek gandor neuraleko zelulekin partekatzen dute migrazio mekanismo hori[14,15]. Tumoreak gertu edo urrutira barreiatu daitezke metastasiak sortuz. Normalean, gertuko azaleretara (satelite edo iragate metastasiak), gongoil linfatikoetara eta larruazalpeko ehunetara barreiatzen dira. Bestalde, urruneko metastasi ohikoenak urruneko larruazala, birikak, burmuina, gibela, hezurra eta hesteetan ematen dira[16].

1.3.3. Melanomaren diagnostikoa eta estadifikazioa

Biziraupen-tasak nabarmen handiagoak dira tumorearen detekzio goiztiarrarekin. Lesioa kirurgikoki garaiz erauzten ez bada eta hazten jarraitzen badu, minbizi hilgarrienetako bat bihurtzen da melanoma. Beraz, detekzio goiztiarrarekin laguntzeko baliabideak izatea garrantzitsua da. Melanoma tumorea identifikatzeko ABCDE irizpideak erabiltzen dira. Ingelesez: Assimetry (asimetria); Border irregularity (ertz irregularrak); Color variation (kolore aldaketa); Diameter (diametroa); Evolution (eboluzioa) (staging melanoma).



Asimetria: oreztak bi erdi ezberdin ditu. Lesio goiztiarrak, oro har, erritmo desberdinean hazten dira, eta, beraz, orezta asimetriko bihurtzen da.

Ertz irregularrak: oreztaren ertzak ez daude ondo zehaztuta, eta irregularrak dira.

Kolore aldaketa: oreztaren barruan kolore aldaketak daude. Tonu marroi edo beltz ezberdinak izan ditzake, edo baita zuria, urdina eta gorria ere.

Diametroa: melanomek 6 mm-tik gorako diametroa izan ohi dute.

Eboluzioa: orezta bat, itxuraz, pazientearen beste oreztekin alderatuta ezberdina denean, edo, denbora batean orezta bera aldatu bada (tamaina, kolorea, forma, etab.), melanoma dela susmatzen da.

Irudia 8. Melanoma goiz detektatzeko ABCDE irizpideen adibideak[20].

Zoritxarrez, ABCDE irizpideek melanoma kasuen %60-65-a bakarrik ondo diagnostikatzen dute, eta, beraz, melanomaren diagnostikoan eta pronostikoan laguntzeko irizpide gehigarriak behar dira. Horregatik, Clark eskala eta Breslow eskala elkarrekin erabiltzen dira.

Clark eskalak tumorearen inbasio-maila eta kaltetutako larruazal-geruzak zehazten ditu. Wallace H. Clarkek 1966an garatu zuen estadifikazio-sistema hori, eta 5 maila desberdin aitortzen dira:

- I: melanoma zelulak epidermisera bakarrik mugatzen dira. Melanoma *in situ* ere deitzen da.
- II: zelulek dermis papilarra inbaditzen dute, epidermisaren azpian dagoena.
- III: melanoma zelulek dermis papilar eta erretikularraren lotura inbaditzen dute.
- IV: melanomak dermis erretikularra edo sakona inbaditzen du.
- V: tumorea dermisaren azpiko larruazalpeko gantzeraino hazten da.

Hala ere, Clark eskala oso gutxitan erabiltzen da gaur egun, iragarpen balio baxua duela frogatu delako, Breslow eskala baino subjektiboagoa eta gutxiago erreproduzitzen delako. Gainera, askotan zaila da Clarken II. eta III. mailen artean bereiztea, eta ezin da oin- eta esku-azpietan agertzen diren melanometan erabili. Beraz, Clark eskalaren erabilera Breslow sakonera 1 mm baino txikiagoa den kasuetara mugatu da[18].

Breslow eskala Alexander Breslowek ezarri zuen 1970ean, eta tumorearen sakonera zehazten du. Erauzitako tumorearen lodiera neurtzen da, eta tumorearen sakoneraren arabera 5 urterako biziraupen-tasa kalkulatzen da. Normalean, Breslow-ren lodiera zenbat eta handiagoa izan, orduan eta okerragoa izaten da pazientearen etorkizuna[18].

- <1 mm: 5 urteko biziraupen-tasa %92-97koa da.
- 1 - 2 mm: 5 urteko biziraupen tasa %80-92koa da.
- 2 - 4 mm: 5 urteko biziraupen tasa %60-75koa da.
- >4 mm: 5 urteko biziraupen tasa %50-koa da.

Metodo hori oso zehatza da melanomaren pronostikoa aurreikusteko, eta, beraz, melanomaren TNM estadifikazio estandarraren sisteman sartu da. TNM estadifikazioa mundu mailan onartutako sistema bat da, minbizi baten etapa alfanumerikoki deskribatzen duenak. TNM sistema Minbiziaren Kontrolerako Nazioarteko Batasunak (UICC) eta Minbiziari buruzko Amerikako Komite Bateratuak (AJCC) garatu zuten, eta ordutik hainbat aldiz eguneratu da, melanomaren ikerketan egindako aurrerapenak direla eta[17]. Sistema hori tumore solido askorentzat erabiltzen da eta derrigorrezko hiru parametroren ebaluazioan oinarritzen da:

T: Tumore primarioaren tamaina eta hedadura deskribatzen ditu. Breslow eskalan oinarritzen da tumorearen lodiera zehazteko eta ultzera presentzia aztertzeko.

N: Tumorea hurbileko gongoil linfatikoetara nola hedatzen den, eta gongoilean dagoen metastasia mikroskopikoa edo makroskopikoa den deskribatzen du. Gainera, iragaitzazko metastasiak ere identifikatzen ditu, hau da, tumore primariotik 3 cm baino gehiagora daudenak baina oraindik gongoil linfatikora iritsi ez direnak. Bestalde, lesio sateliteak ere identifikatzen ditu, 0,5 mm baino gehiagoko tumoreak, tumore primarioaren ebakidura histologiko berdinean daudenak.

M: Urrutiko metastasien presentzia deskribatzen du.

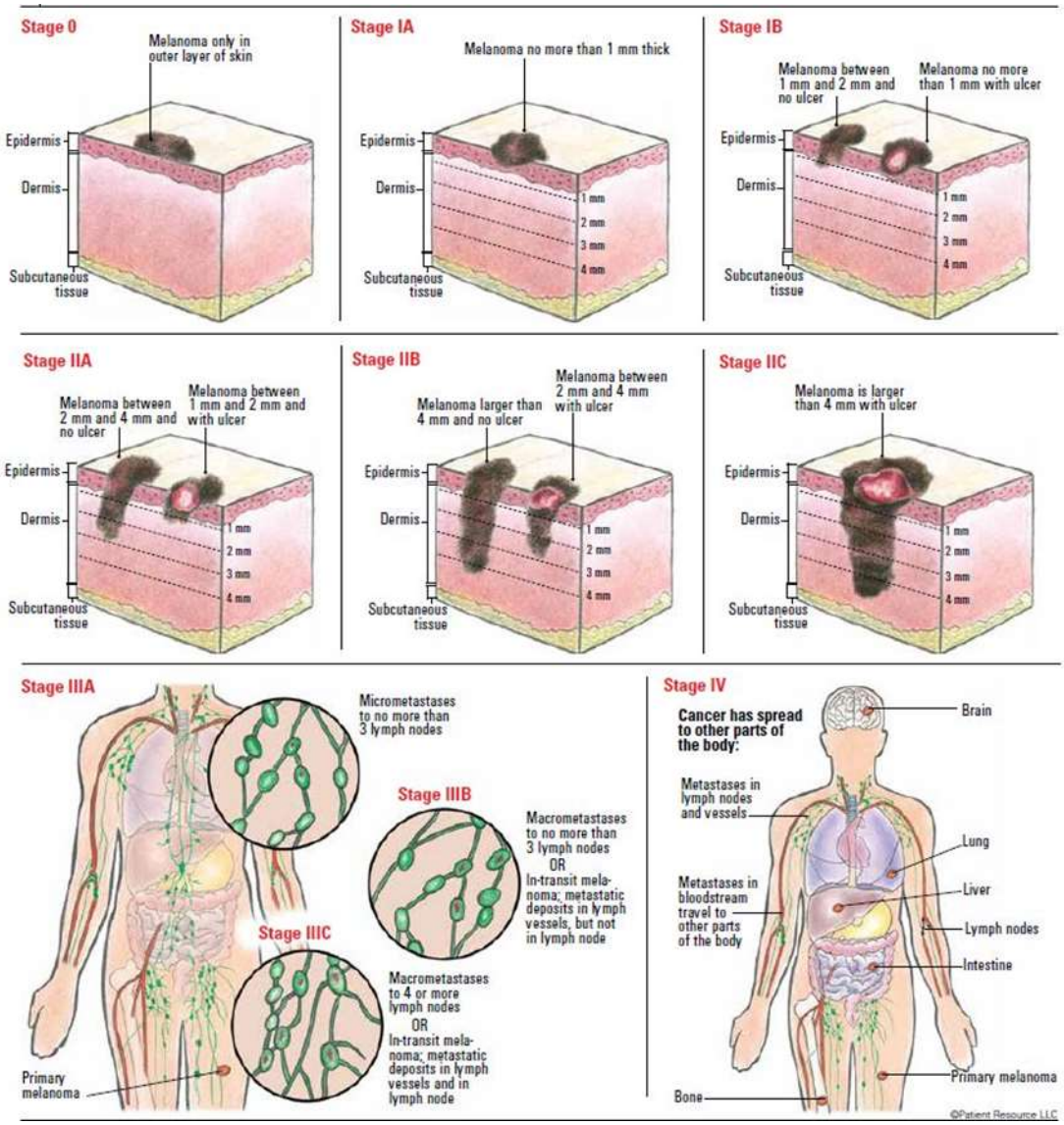
Taula 1. Melanomaren estadifikazioa sailkatzeko erabiltzen diren irizpideen deskribapena[17].

Estadioa	Deskribapena
Tumorea (T)	
Tx	Tumore primarioa ezin da ebaluatu
T0	Tumore primarioa ez da detektatzen
Tis	Melanoma *in situ*. Minbizi-aurreko lesioa. Melanoma zelulak epidermis eta dermisaren artean kokatzen dira, baina oraindik ez dituztegeruza horiek inbaditu
T1	Tumorearen lodiera ≤1 mm
T1a	Tumorearen lodiera ≤1 mm, ultzeraziorik ez eta tasa mitotikoa < 1/mm^2
T1b	Tumorearen lodiera ≤1 mm, ultzerazioa edo tasa mitotikoa < 1/mm^2
T2	Tumorearen lodiera 1-2 mm
T2a	Tumorearen lodiera 1-2 mm, ultzeraziorik ez
T2b	Tumorearen lodiera 1-2 mm, ultzerazioa bai
T3	Tumorearen lodiera 2-4 mm
T3a	Tumorearen lodiera 2-4 mm, ultzeraziorik ez
T3b	Tumorearen lodiera 2-4 mm, ultzerazioa bai
T4	Tumorearen lodiera >4 mm
T4a	Tumorearen lodiera >4 mm, ultzeraziorik ez
T4b	Tumorearen lodiera >4 mm, ultzerazioa bai
Gongoil linfatikoa (N)	
Nx	Inguruko gongoilak ezin dira ebaluatu
N0	Ez dago melanomarik inguruko gongoiletan
N1	Melanoma gongoil 1-ean aurkitu da
N1a	Melanoma gongoil 1-ean aurkitu da, metastasi mikroskopikoak
N1b	Melanoma gongoil 1-ean aurkitu da, metastasi makroskopikoak
N2	Melanoma 2-3 gongoiletan aurkitu da
N2a	Melanoma 2-3 gongoiletan aurkitu da, metastasi mikroskopikoak
N2b	Melanoma 2-3 gongoiletan aurkitu da, metastasi makroskopikoak
N2c	Iragate edo satellite melanoma lesioak aurkitzen dira, baina ez da metastasirik aurkitzen gongoil linfatikoetan
N3	Melanoma ≥4 gongoil linfatikoetan aurkitzen da, edo bateratuta dauden ≥2 gongoiletan Iragate edo satelite melanomak aurkitzen dira, gehi gongoil linfatikoetan metastasiak
Metastasiak (M)	
Mx	Metastasiak ezin dira ebaluatu
M0	Ez dago metastasirik
M1a	Metastasiak larruazalean, larruazalpeko ehunean edo urruneko gongoil linfatikoetan
M1b	Metastasiak biriketan
M1c	Metastasiak urrutiko beste edozein organoetan

Melanoma TNM sistema erabiliz sailkatu ondoren, gaixotasunaren etapa orokor bat esleitzen da, non 0 etapa *in situ* melanoma den eta minbizi-aurrekoa bezala ezagutzen den. I. eta II. etapak lesio lokalizatutzat hartzen dira. III. fasea inguruko gaixotasunari dagokio, eta IV. fasea gaixotasun aurreratutzat jotzen da[17,19].

Taula 2.TNM sisteman oinarritutako melanomaren estadifikazioa[17].

Estadioa	T	N	M
0	Tis	N0	M0
IA	T1a	N0	M0
IB	T1b T2a	N0	M0
IIA	T2b T3a	N0	M0
IIB	T3b T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T4a	N1a N2a	M0
IIIB	T1-T4b T1-T4a	N1a N2a N1b N2b N2c	M0
IIIC	T1-T4b Any T	N1b N2b N2c N3	M0
IV	Any T	Any N	M1



Irudia 9. Melanomaren garapen-etapa bakoitzaren deskribapen klinikoa[17].

1.3.4. Melanomaren sailkapen klinikoa

Klasikoki, kokapen anatomikoaren eta eboluzioaren arabera, klinikoki lau melanoma mota bereizi dira.

- **Azalerako hedadura duen melanoma (SSM):**

Kaukasoarren artean melanoma mota ohikoena da, talde etniko horretako melanoma kasu guztien %70-80 delarik. Normalean, eguzkiarekin kontaktuan dagoen azalean sortzen da, hala nola, enborra eta bizkarra gizonezkoetan eta bizkarra eta beheko gorputz-adarrak emakumezkoetan. Gainera, SSM-en %40 inguru, aurretik zegoen lesio batetik eboluzionatuz sortzen dira, adibidez, nevus arrunt edo displasiko batetik. Lesio horren ezaugarri klinikoek ABCDE irizpideak betetzen dituzte; izan ere, oro har, ertz irregularrak eta asimetrikoak ditu, kolore-aldakuntzarekin, eta 6 mm-tik gorakoa izanez. Melanoma mota horrek hazkuntza-fase erradial luzea du, non lesioa mehe mantentzen den melanozitoak epidermisaren barruan hazten baitira. Hala ere, tumorea erauzten ez bada, hazkuntza bertikalaren fasea has daiteke eta dermisa inbaditu eta hipodermisera irits daiteke, pazientearen biziraupena arriskuan jarriz[19–21].

- **Melanoma nodularra (NM):**

Melanomaren bigarren motarik ohikoena izan arren (% 15-25eko intzidentzia), formarik erasokorrena bezala identifikatzen da, hazkuntza bertikal fasean zuzenean hasten den hazkuntza azkarra baitu. Gainera, lesio berri batetik garatzen da, ez du aurretik zegoen lesiorik behar, eta, beraz, zailagoa da identifikatzea. Askotan, jada ahalmen metastatikoa duenean diagnostikatzen da. Sarritan adin ertaineko gaixoen buruan, lepoan edo enborrean agertzen da, eta ohikoagoa da gizonezkoetan emakumezkoetan baino. Makroskopikoki, kolore beltz urdinxkako lesio gisa deskriba daiteke, hazkuntza azkarrekoa, kupula formakoa, ultzeraziorako joera handia duena[20].

- **Lentigo-maligna melanoma (LMM):**

Melanoma kasu guztien %10 baino gutxiago dira. *In situ* existitzen den melanoma batetik sortzen da, lentigo-maligna izenekoa, epidermisean dagoena eta periferikoki hazten dena; izan ere, etapa honetan 5-7 cm-ko diametroa har dezake. Urte batzuetan, lesioak fenotipo inbaditzailea hartzen du eta bertikalki hazten da; aurrerantzean, melanoma lentigo-maligna deiturik. Eguzkiaren bitartez kalteturik dauden larruazaletan sortzen da, batez ere kaukasoar adinduen aurpegi eta lepoetan. Lesio handia izaten da (> 3 cm), kolore anitzekoa (marroi tonu ezberdinak, beltzak) eta eremu altuak dituena[20].

- **Melanoma akral lentiginosoa (ALM):**

Beste motak ez bezala, ALM ohikoagoa da beltzaranetan (%60-70) eta asiarretan (%35-45), azal argiko banakoetan baino (<% 5). Gorputz-gainazal ez iletsuetan gertatzen da, hala nola esku- eta oin-azpietan, eta azazkalen azpian, nahiz eta ALM gehienak adineko pertsonen oin-azpietan garatzen diren. Bere itxura, melanoma lentigo-malignaren antzekoa da, beltzak, irregularrak eta handiak izanik.

Bestalde, hain ohikoak ez diren beste aldaera batzuk ere badaude: spitzoid melanoma, zelula txikien melanoma, nevus urdin gaiztoa, melanoma desmoplasikoa, begietako melanoma (konjuntiba edo ubeala) eta mukosetako melanoma, besteak beste.

1.4. Melanoma tratamenduak

Melanoma duten pazienteen errekuperaziorako behar den terapia, diagnostikoa egin deneko etapa edo mailaren arabera aukeratzen da. Errekuperazio-tasa ia %100ekoa da goiz diagnostikatzen denean, baina melanoma metastatikoarentzat ez dago terapia guztiz eraginkorrik. 0 eta II arteko estadioetan aurkitutako melanometarako aukerako tratamendua erauzketa kirurgikoa da, eta ebakuntzan tarte handi bat uzten da aurrerantzean tumore berria ager ez dadin[22,23]. Gainera, Breslowren lodiera handia bada eta susmo metastatikoa badago, gongoil linfatiko zentinelaren biopsia egiten da. Hori, tumore primarioaren lehen drainatze-gongoila bezala definitzen da, eta, beraz, zelula metastatikoak jasotzen lehena izango litzateke. Abordaje kliniko horrek gongoil linfatiko hori identifikatzea eta ondoren erauztea ahalbidetzen du. Gero histologikoki aztertzen da, eta zelula metastatikoak aurkituz gero, berehalako linfadektomia egiten da.

Melanomak erradioterapiarekiko erresistentetzat hartu izan da betidanik, erradioterapiaren erabilera tratamendu aringarrirako erabili izanik. Hala ere, erradioterapia terapia laguntzaile gisa aplika daiteke kirurgiaren ondoren, edo kirurgian iradokitako erauzketa-marjinak aplikatu ezin direnean, bereziki melanoma lentigo-malignoa duten adineko pazienteetan[24].

1.4.1. Melanoma metastatikoa

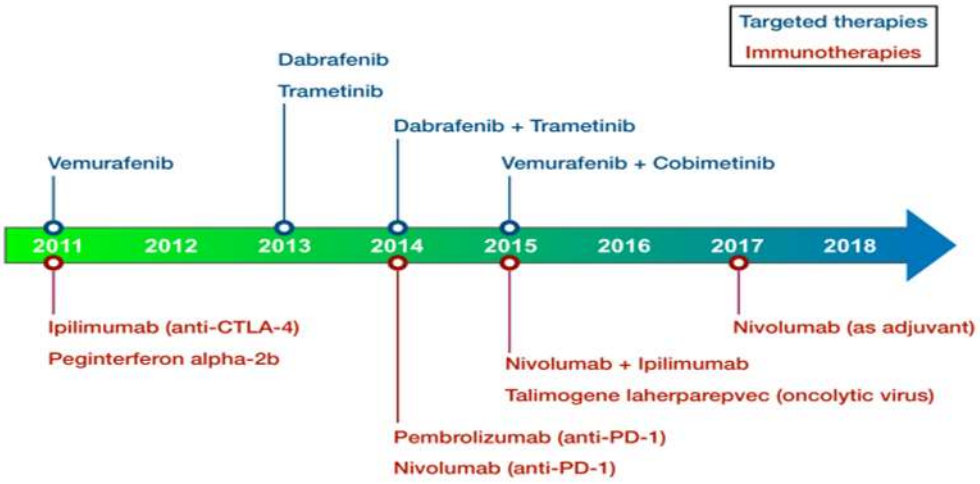
Paziente metastatikoen tratamenduak melanomaren kudeaketan gainditu beharreko oztopo nagusia izaten jarraitzen du. Oso pronostiko pobrea dute gaixo horiek, kirurgia eta erradioterapia ez baitira eraginkorrak gaixotasun aurreratuan. Dacarbazina bidezko kimioterapia lehen mailako tratamendua izan da 1975 eta 2011 artean. Orain dela gutxira arte, FDA-k (U.S. Food and Drug Administration) melanoma metastatikoarentzat onartutako tratamendu bakarra dacarbazina zen, baina gaur egun temozolomida ere kimioterapia gisa erabiltzen da. Hala ere, erantzun-tasa baxua dute, eta farmakoaren eragina 5-6 hilabetez soilik irauten du[25–27].

Melanoma metastatikoa tratatzeko beste aukera bat immunoterapia da, eta gaixoaren sistema immunea bera erabiltzen du tumorea erasotzeko. Melanoma tratatzeko onartutako lehen agente immunomodulatzailea IL-2 zitozina izan zen 1998an. Oro har, terapia laguntzaile gisa ematen da, kimioterapia edo erradioterapiarekin batera. Hala ere, toxikotasun handia du, eta, beraz, egoera onean dauden pazienteetan baino ez da erabiltzen[25]. Interferon- α (IFN-α) eta peginterferon α-2b-a ere erabiltzen dira, nahiz eta erantzun antitumoral baxua duten eta albo-ondorio larriekin lotzen diren; beraz, soilik IV. faseko pazienteetan erabili dira[25,28].

Melanoma aurreratuentzako tratamenduen iraultza 2011n hasi zen, melanoma metastatikoa tratatzeko ipilimumab-en erabilera onartu zenean. Hori ere terapia laguntzaile gisa erabiltzen da, tumorea erauzita duten III. estadioan dauden pazienteetan. Antigorputz monoklonal humanizatu horrek T linfozito zitotoxikoei lotutako proteina 4-aren (CTLA-4) aurka egiten du, eta horren ondorioz IL-2-a jariatzen da, T zelula zitotoxikoak aktibatuz eta horien hazkuntza sustatuz[25,28–30].

Garatu diren beste inhibitzaile batzuk, PD-1 bidea inhibitzen dute. 2015ean, FDA-k nivolumab eta pembrolizumab onartu zituen melanoma aurreratuan edo terapia lagungarri moduan erabiltzeko kirurgiaren ondoren. Antigorputz monoklonal horiek T zelulen PD-1 hartzailearen eta melanoma zelulen PD-L1 antigenoaren arteko elkarrekintza blokeatzen dute, eta horrek T zelulen erantzun antitumorala areagotzen du. Bestalde, ipilimumab-aren eta nivolumab-aren konbinazioa aztertu da, baina albo-kalte garrantzitsuak deskribatu dira[25,28–32].

Melanoma tratatzeko beste aukera terapia gidatua da. Kasuen %50ean, BRAF-V600E mutazioa agertzen da. Beraz, FDA-k eta EMA-k bi BRAF-V600E inhibitzaile desberdinak onartu dituzte melanoma metastatikoan erabiltzeko, horiek, vemurafenib 2011n eta dabrafenib 2013an onartuak izan dira. Pazienteetan hobekuntza nabarmena erakutsi duten arren, tratamenduarekiko erresistentzia sortzen dute eta efektuak ez du asko irauten[29,33]. Horretaz gain, monoterapiarako onartutako MEK inhibitzaileak trametinib eta cobimetinib dira, eta BRAF edo NRAS ohiko mutazioak dituzten pazienteekin erabil daitezke. Nabarmenki, MEK eta BRAF inhibitzaileak konbinatuz emaitza sinergiko oso eraginkorra sortzen da[25,29,33]. Une honetan onartuta dauden terapia konbinatuak [vemurafenib eta cobimetinib] eta [dabrafenib eta trametinib] dira[32,34].



Irudia 10. Melanoma tratatzeko aukeren kronologia 2011tik[34].

1.4.2. Aukera terapeutiko berriak

Azkenaldian, hainbat proba kliniko egin dira melanoma tratatzeko agente berrien eraginkortasuna aztertzearren. Aukera terapeutiko berri horietako batzuk birus onkolitikoak erabiltzen dituzte, eta Talimogene laherparepvec (TVEC) da orain arte onartutako aukera bakarra. Birusak minbizi zelulen barruan erreplikatzen dira, GM-CSF sortuz eta zelula neoplasikoen lisia eraginez[29].

Aztertzen ari den estrategia interesgarri bat minbiziaren aurkako txertoak dira, pazientearen immunitate-sistema estimulatzen baitute, tumorearen aurka jardun dezan. Orain arte aztertutako txertoak gp-100, Melan-A eta tirosinasa bezalako antigenoen aurka diseinatuak izan dira. Hala ere, erantzun-tasa baxuak izan dituzte[35].

Ikertzen ari den beste tratamendu-aukera bat adopziozko terapia zelularra da, isolatutako tumore-linfozito infiltratuak (TIL) erabiltzen dituena, eta ezaugarri antitumoral indartsuak dituztenak. TIL-ak *ex vivo* hazten dira eta berriro gaixoan txertatzen dira metastasi ezberdinak tratatzeko[35]. Beste ikerketa batean, linfozito autologoak genetikoki eraldatu zituzten, melanoma zelulak detektatu eta erasotzeko. Etorkizun handiko emaitzak izan arren, tratamendu-aukera horrek ikerketa gehiago behar du tumore-zelulekin lotura espezifikoagoa lortzeko[36].

1.5. Melanomaren biomarkatzaileak

Melanomaren diagnostikoan eta tratamenduan aurrerapen garrantzitsuak lortu diren arren, ez da aurkitu gaixotasun horretarako terapia edo biomarkatzaile guztiz eraginkorrik. Beraz, biomarkatzaile berriak aurkitzeko premia izugarria dagoela iradoki da. Bilatzen diren markatzaileak iragarpenean, diagnostiko goiztiarrean eta pronostikoan lagundu edo itu terapeutikoak izan litezke.

Biomarkatzaile bat objektiboki neur daitekeen substantzia, egitura edo prozesu biologiko bat da, gorputzean edo hondakin produktuetan dagoena. Gainera, gaixotasun jakin bat pairatzeko arriskua, presentzia, egoera eta izan dezakeen pronostikoa adierazteko gai izan behar du edo terapiarekiko sentikortasuna zehaztu. Biomarkatzaile horiek, adibidez, bidezidor genetikoak edo gene partikularrak, metabolismo bideak, proteinak edo lipidoak izan daitezke, kartzinogenesi-prozesuak sortutako alterazio genetiko, zelular, biokimiko edo molekularren ondorioz aldatzen direnak[37].

Minbizian erabiliko den biomarkatzaile ideal batek tumorea garatzeko prozesuan parte hartu behar du, eta, itu terapeutikoa ere izan. Hortaz, bere adierazpen mailen aldaketak gaixotasunaren etapako aldaketekin edo tratamendu arrakastatsuarekin korrelazioan jarri beharko lituzke. Gainera, ez litzateke ehun edo banako osasuntsuetan agertu behar, eta minbizi mota jakin baterako espezifikoa izan beharko luke. Modu idealean, biomarkatzaile hori minbizi mota jakin batekiko, diagnostiko goiztiarrarekiko, pronostikoarekiko eta medikamentuekiko erantzuna ebaluatzeko erabiliko litzateke. Gainera, erraz eta modu ez inbaditzailean antzeman beharko litzateke.

Bereziki melanoman, diagnostikoa, behaketa eta identifikazio klinikoan oinarritzen da. Horren ostean, baieztapen histopatologikoa egiten da, hurrengo biomarkatzaile histologikoak aztertuz:

HMB45 antigorputzak nevus eta melanoman dagoen aurre-melanosoma proteina (**Pmel**) ezagutzen du. Haatik, melanoma desmoplasiko gaiztoetan sentikortasun gutxi erakutsi du.

Melan-A proteina melanozitoen eta melanomaren zitoplasman aurkitzen da. Melanoma eta tumore ez-melanozitikoak bereizteko espezifikotasun handia du, baina ez da hain sentikorra melanoma metastatikoa zein melanoma desmoplasikoa hautemateko, horiek tumore primarioekin alderatzean.

Tirosinasa entzima melaninaren ekoizpenean inplikatuta dago, eta melanoma eta tumore ez-melanozitikoak bereizteko oso eraginkorra da. Hala ere, melanoma desmoplasikoa detektatzeko sentikortasun txikia erakusten du.

S100B proteina biomarkatzaile immunohistokimiko gisa erabili ohi da melanoma identifikatzeko, melanoma desmoplastikoak detektatzeko sentikortasun handia erakusten baitu. Aitzitik, ez da melanoman soilik detektatzen. Izan ere, giltzurruneko eta gibeleko kaltea duten pazienteetan, beste tumoreen ondorioz sortutako gibeleko metastasietan, eta hainbat infekzio- eta hantura-nahasmendu dituzten pazienteetan ere adierazpen maila altua erakusten du.

Markatzaile horiek melanomaren diagnostiko histopatologikoa baieztatzen laguntzen duten arren, horietako bakar batek ere ezin ditu bereizi lesio melanozitiko gaiztoak eta ez-gaiztoak.

Horietaz gain, beste biomarkatzaile batzuk ere badaude, estadio aurreratuagoetara aurrera egiteko probabilitate handiagoa duten edo tratamendu jakin baten onuradun izango liratekeen pazienteak zehazten laguntzen dutenak.

Biomarkatzailetzat jo ezin diren arren, Breslow lodiera eta tasa mitotikoa dira melanomaren pronostikoa eta pazienteen bizi-raupena zehazteko faktore zehatzenak, zeinak TNM estadifikazio sistemaren parte diren. Izan ere, Breslow lodiera handia eta tasa mitotiko altua duten tumoreek, infiltratutako linfozitorik ez izatearekin batera, gongoil linfatiko-zentinela positiboa izateko joera handiagoa dute.

Bestalde, **BRAF-V600E** mutazioaren presentzia aztertzeak pazienteentzako tratamendu egokia zehazten laguntzen du, vemurafenib bidezko tratamenduak mutazio hori duten pertsonen bizi-raupena nabarmen handitzen duela frogatu baita.

Markatzaile histopatologikoez gain, biomarkatzaile serikoak ere badaude, detektatzeko errezagoak direnak. Horien artean, **laktato deshidrogenasa** (LDH) entzima dago. Serumeko LDH maila altuak melanoma metastatikoarekin lotzen dira, bereziki gibeleko metastasiarekin. Izan ere, TNM estadifikazio sisteman pronostiko txarreko markatzaile gisa eta terapiari erantzuteko iragarle negatibo gisa sartu da. Hala ere, ez da bakarrik melanoman detektatzen[38].

Badira beste biomarkatzaile batzuk, melanomarentzat esklusibo izan ez arren, pronostikoaren ebaluazioan erabiltzen direnak. Hainbat hantura-prozesuetan horien mailak handituta agertzen dira, hala nola, tumore-hanturan, angiogenesian eta metastasian. Horien artean VEGF, metaloproteinasak eta ziklooxigenasa-2 entzima daude, besteak beste[39].

1.5.1. Biomarkatzaile berrien aurkikuntza

Biomarkatzaile berriak aurkitzeko ikerketa-prozesua hiru fasetan bana daiteke. Lehenengoa oinarrizko azterketetan oinarritzen da, eta aurkikuntza fasea deritzo. Hemen, hipotesia eta ikuspegi analitikoa definitzen dira, eta proposatutako saiakuntzak gauzatzen dira. Fase horretan biomarkatzaile potentzialak identifikatzen dira. Hautagai sendoak aurkitzeko, erabilitako prozesu analitikoak zehatzak eta erreproduzigarriak izan behar dira, laborategi ezberdinetan emaitzen erreproduzigarritasuna berma dadin.

Bigarren fasean, biomarkatzaileen baliozkotasun analitikoa eta balidazio klinikoa aztertzen dira. Horretarako, biomarkatzaileak klinikan erabiliko liratekeen plataforman inplementatzen dira. Gero, lagin berriak aztertzen dira, eta biomarkatzaileak laginak fidelki sailkatu beharko lituzke balioztatze-talde egokietan.

Biomarkatzaile batek aldeko balidazio analitikoa eta klinikoa lortu badu, prest dago haren erabilgarritasun klinikoa zehazteko. Fase honetan, eraginkortasun klinikoa onura/kalte erlazioarekin batera ebaluatzen da. Pazientearen arretan erabiliko litzatekeen biomarkatzaile batek ebidentzia kliniko handia izan behar du. Gainera, inplementazio klinikorako, biomarkatzaileak erregelamendu bidezko onarpena, merkaturatzea eta praktika klinikoko gidetan sartzea lortu beharko du[37,40].

1.6. Melanomaren ezaugarri bereizgarriak

Minbizia bereizteko gai diren biomarkatzaile berriak identifikatzeko, tumoreen izaera konplexua ondo ulertu behar da. Helburu horretarako, minbiziaren ezaugarriak zehaztu ziren. Minbizi-zelulek 10 gaitasun desberdin eta osagarri bereganatzen dituzte, minbizi zelulen biziraupena, proliferazioa eta barreiadura bermatzeko, edozein minbizi mota izanda ere[41]. Kontzeptualizazio sinplifikatu horrek minbizi mota desberdinen biologia sofistikatua ulertzen laguntzen du. Ezaugarri horiek tumore-zelulei zein estromari eragiten diete, eta horrela ezartzen dira:

- **Aldakortasun genomikoa:** melanoma zeluletan, izpi ultramoreek ezegonkortasun genomikoa eragiten dute, mutazioen agerpena eraginez, minbiziaren beste ezaugarri bereizgarri batzuk garatzea ahalbidetuz, eta, modu horretan tumoreak aurrera eginez[42].
- **Minbiziaren aldeko hantura:** tumore-ehunek hantura kronikoa dute, eta, horri esker, zelula immuneek seinaleztapen molekula asko isurtzen dituzte. Askatutako molekula horiek minbizi zelulen hazkuntza sustatzea eta apoptosia ekiditzea eragiten dute, ezegonkortasun genomikoari lagunduz eta angiogenesia eta metastasia suspertzen dute[41]. Melanoman, zehazki, erradiakzio ultramoreak melanozitoak eta keratinozitoak kaltetzen ditu, zeintzuk HMGB1 zitozina askatzen duten, neutrofiloak tumore-mikroingurunean bilduz[42]. Zelula immune horiek genomaren ezegonkortasuna, angiogenesia eta metastasia sustatzen duten hanturaren-aldeko proteinak askatzen dituzte.
- **Proliferazioa sustatzen duen seinaleztapena:** minbizi zelulek hazkuntza faktoreak sortzen eta askatzen dituzte, eta ligando horien hartzaileen adierazpena ere handitzen dute. Gainera, estromako zeluletara seinaleak bidaltzen dituzte, hazkuntzari eusteko molekula gehiago sor ditzaten. Bestalde, proliferazioa areagotze hori hazkuntza-faktore gabe ere lor dezakete, jarduera horiek sustatzen dituzten seinaleztapen bideak aktibatuz. Melanoman, askotan, BRAF-en mutazio aktibatzailearen ondorioz lortzen da hori, RAS/RAF/MERK/ERK bidea etengabe aktibatuta mantentzen duelako, eta hori gehiegizko hazkuntzan itzultzen da[42].
- **Tumoreen hazkuntza zailtzen duten faktoreak ekidin:** tumoreen hazkuntza galarazten duten bi proteina, p53 eta pRb dira. Horiek, ziklo zelularra gelditzen dute eta seneszentzia edo apoptosia eragiten dute. Melanoman, CDKN2A genearen aurkako mutazio desaktibatzaileak ohikoak dira. Gene horrek, pRb defosforilatu dezakeen p16 proteina kodetzen du, eta, ondorioz, desaktibatu eta ziklo zelularra geldiarazi. CDKN2A mutatuta badago, p16-ak ez du pRb desaktibatzen, eta ziklo zelularra etengabe aktibatuta dago[42].
- **Heriotza zelularra saihestu:** apoptosia funtsezkoa da ehunen homeostasirako. Bide apoptotikoetan akatsen bat izanez gero, zelulek heriotza zelularraren aurkako erresistentzia sor dezakete. Larruazaleko melanoman, bide apoptotikoetako akatsak deskribatu dira. Adibidez, melanoma metastatikoan, Apaf-1 proteinaren adierazpena galtzen da. Proteina horrek, apoptosiaren aktibazioan funtsezko papera betetzen du, DNA kaltetzearen ondorioz c zitokromoa mitokondriatik askatzen denean[42].
- **Hilezkortasuna sustatu:** minbizi zelulek etengabe zatikatzeko gaitasuna dute. Hori telomerasaren gainadierazpenaren ondorioz ematen da, zeinak etengabe DNA telomerikoa gehitzen dieten telomeroen muturrei. Hortaz, ez dira laburtzen eta ez da seneszentzia edo apoptosia ematen[41].
- **Angiogenesia bultzatu:** Angiogenesia ezinbestekoa da minbiziek aurrera egin eta barreiatzeko. Melanoma-zelulek angiogenesian laguntzen duten glukoproteinak askatzen dituzte[42].

- **Inbasioa eta metastasiaren aktibazioa:** melanoma zelulek hainbat proteina sortzen dituzte, metaloproteinasak kasu, ehunen xafla basala eta matrize estrazelularra degradatzen laguntzen dutenak, prozesu metastatikoa erraztuz[42].
- **Suntsipen immunea saihestu:** berezko eta lorturiko immunitate-sistemek funtzio garrantzitsua dute tumore-zelulak desagerrarazteko. Minbizi zelula batzuk, melanoma zelulak kasu, immune-sistematik ihes egin eta horrek eragindako suntsipena ekidin dezakete[42].
- **Zelulen-energia kudeaketaren deserregulazioa**: minbizi zelulek dituzten behar guztiak asetzeko, euren metabolismoa egokitzen dute, behar duten hazkuntza eta zatiketa-tasa altuei eusteko. Melanoma zelulek fosforilazio oxidatibotik glukolisira aldatzen dute metabolismoa, eta glukosa xurgapena nabarmen handituta dago[42]. Horrez gain, beste bide anaboliko eta kataboliko batzuk ere moldatzen dira minbizi zeluletan. Horien artean, lipidoen metabolismoak garrantzi handia hartu du azken urteotan.

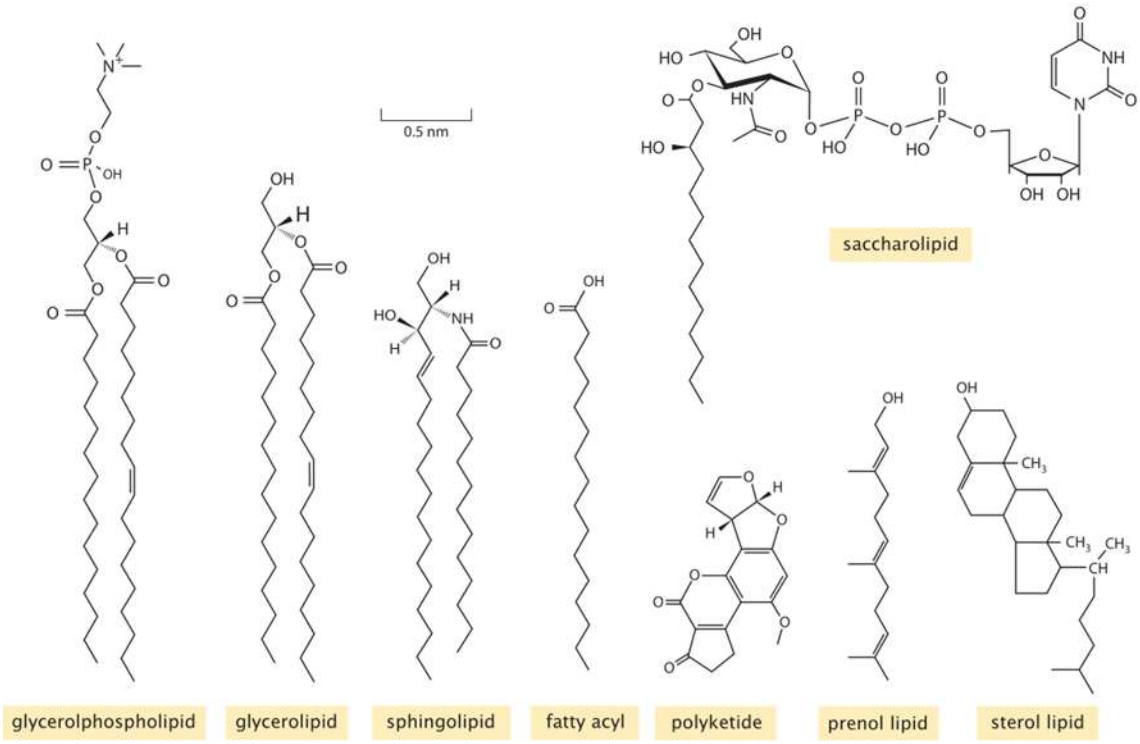
2. Lipidoak, zelula-metabolismoa eta minbizia

2.1. Lipidoak

Lipidoak zeluletako oinarrizko prozesu fisiologiko ugaritan parte hartzen duten molekulak dira. Laburbilduz, zelulen seinaleztapen bidezidorretan, energiaren biltegiratzean, mintz biologikoetako egitura-euskarrian eta fisiologikoki garrantzitsuak diren molekulen sintesian parte hartzen dute, hala nola behazun-azidoen, hainbat hormonen eta bitaminen sintesian. Beraz, ugalketa, migrazioa, biziraupena eta heriotza bezalako prozesu zelularrak kontrolatzen dituzte, guztiak ere prozesu kantzerigenoarekin lotura estua izanik.

2.1.1. Lipidoen sailkapena

Milaka lipido molekula ezberdin egon arren, guztiek ezaugarri komunak dituzte. Esaterako, uretan disolbagarritasun gutxi edo bat ere ez, baina disolbagarritasun ona disolbatzaile polarretan. Oro har, molekula hidrofobiko edo anfipatiko gisa deskribatzen dira. Egitura molekularraren arabera, zortzi klasetan sailka daitezke: gantz-azidoak, glizerolipidoak, glizerofosfolipidoak, esfingolipidoak, esterol lipidoak, prenol lipidoak, sakarolipidoak eta poliketidoak.



Irudia 11. Lipido mota ezberdinen egitura orokorra[46].

Gantz-azidoak (FA) molekula lipidiko sinpleenak dira, eta era berean lipido biologikoen klase funtsezkoenetako bat, lipido konplexuenentzat eraikuntza-bloke gisa balio baitute. Bere egitura bi zatitan bana daiteke. Alde batetik luzera ezberdineko hidrokarburo apolar kate bat dute, karbono kopuru bikoitia izaten duenak, eta bestetik, muturrean azido karboxiliko polar talde bat. Hidrokarburo katearen barruan lotura bikoitzak eta hirukoitzak egon daitezke FA asegabeak eratuz. Horiek FA-monoinsaturatuak (lotura bikoitz edo hirukoitz bakarra; MUFA) edo FA-poliinsaturatuak (insaturazio bat baino gehiago; PUFA) izan daitezke. Insaturaziorik ez badago,

gantz-azido horiek FA aseak izendatzen dira. Azil-kateetan insaturazioak egoteak okerdura bat sortzen du molekularen egituran. 1000 egitura-aldaera baino gehiago identifikatu dira FA-etan, kate-luzera ezberdin, insaturazioen kopuruaren eta posizioaren, eta hidrokarburoen katean zehar dauden zenbait ordezkatzaile gehigarriren konbinazioaren arabera. Hala ere, naturan ohikoenak 20 inguru dira; azido palmitikoak (16:0), oleikoak (18:1) eta linoleikoak (18:2) FA arruntenen %80 inguru izanik[43].

Taula 3. Gantz-azido molekula arruntenen deskribapena.

Gantz-azidoa	Karbono kopurua	Insaturazio kopurua
Azido Palmitikoa	16	0
Azido Oleikoa	18	1
Azido Linoleikoa	18	2

Lipidoak zelularen barruan *de novo* sintetizatu daitezke edo zelula-kanpoko espaziotik xurgatu[44]. Zelulek, FA-k bi mekanismo ezberdinen bitartez xurgatu ditzakete: alde batetik, garraiatzaileen bidezko xurgapena, adibidez, CD36 gantz-azidoen translokasaren bitartez, eta beste aldetik, iragazketa pasiboaren bitartez[45]. Sintetizatu ezin diren eta funtzio biologiko batzuetarako funtsezkoak diren gantz-azidoei esentzialak deritze, eta, beraz, dietatik hartu behar dira. Horiek, FA poliinsaturatuak dira, eta lotura bikoitza metilo muturretik gertu dute. Bi serietan banatzen dira: azido linolenikoan oinarritutako omega-3 seriea osatzen duten lipidoek, 3 eta 4 karbonoen artean lotura bikoitza dutenak; eta azido linoleikoan oinarritutako omega-6 serieak, 6 eta 7 posizioen arteko lotura bikoitzarekin. Lipido horiek xurgatu ondoren, aldaketa metaboliko ezberdinak gertatzen dira elongasen eta desaturasen bidez araututa, beste FA ezberdinetan bihurtuz, azido eikosapentaenoikoa (EPA; omega-3) eta azido arakidonikoa (AA; omega-6) kasu[46].

Bestalde, FA-k berriki sintetiza daitezke malonil-CoA-tik datozen bi karbonodun unitate baten eta azetil-CoA molekula baten kondentsazioaren bidez, azetil-CoA karboxilasa (ACC) eta gantz-azido sintasa (FAS) entzimen eraginez, azido palmitikoa sortuz (16:0). Beste FA ezberdinak sintetizatzeko, *de novo* eratutako azido palmitikotik edo zelula kanpoaldetik xurgatutako FA-etatik abiatuta, lipido horien azilo-katea luzatu eta lotura bikoitzak sartu daitezke. Karbono katearen luzapena elongasa ezberdinen (ELOVL) bitartez ematen da, ziklikoki bi karbonoko unitateak gehitzen dituztenak. Gainera, FA-k kate ase lineal bat izan dezake, edo lotura bikoitz bat edo gehiago izan ditzake. Ugaztun zelulek desaturasa espezifikoak dituzte, eta lotura bikoitzak sartzen dituzte kate luzeko gantz-azidoaren posizio zehatzetan. Bi desaturasa-familia desberdin daude: estearoil-CoA desaturasek (SCD) lotura bikoitza karbono-katearen C9-an sartzen dute, eta GA desaturasek (FADS) lotura bikoitza C5 eta C6 posizioetan sartzen dute[47].

Gantz-azidoa sintetizatu ondoren, CoA molekula bati modu kobalentean lotzen zaio gantz azil-CoA sintetasen (ACS) eraginez, zelula barruko lipido bioaktiboen erreserban sartzeko. FA-ek bi funtzio nagusi dituzte. Alde batetik, beste molekula batzuetara erantsi daitezke lipido molekula konplexuagoak sortzeko. Eratutako lipidoak zelulen barruan hainbat helburutarako erabil daitezke: seinaleztapen lipido-molekulen aitzindari izateko, triglizeridoak sortu energia biltegi gisa erabiltzeko, fosfolipidoak sortu zelula-mintzetan egitura-helburuetarako erabiltzeko eta proteinak palmitoilazioaren bidez eraldatzeko, besteak beste[48,49]. Lipido konplexuenen egituran FA ezberdinen konbinazioak lipido konplexu horien egituran aldaerak sortzen ditu, horien funtzio biologikoa eraginez. Biologikoki garrantzitsuak diren FA batzuek seinaleztapen-molekula

gisa ere jarduten dute. Horien artean, azido arakidonikoaren (20:4) edo beste PUFA-ren oxidazioak eikosanoideak sortzen ditu, prostaglandinak, leukotrienoak eta tronboxanoak barne, berebiziko garrantzi biologikoa duten funtzioak dituztenak.

Beste alde batetik, mitokondrioetan oxidatu eta energia iturri bezala ere erabili daitezke gantz-azidoak.

Glizerolipidoak (GL) glizerol molekula batean bat, bi edo hiru gantz-azido esterifikatuz sortzen dira., horrela, mono-(MG), di-(DG) edo triglizeridoak (TG) sortuz. Bereziki, energia biltegi moduan funtzionatzen dute. Izan ere, gorputzean soberan dagoen energia biltzeko triglizeridoak sortzen ditu, horien oxidazioaren ondorioz energia sortzeko gorputzak behar duenean. Bestalde, DG-ak seinaleztapen-molekulak dira. Konkretuki, bigarren mezulariak dira, eta seinaleak mintzetan zehar transmititzen dituzte. Bestalde, TG-en eta glizerofosfolipidoen sintesian ere parte hartzen dute.

Glizerofosfolipidoak (GPL) zelula-mintzen osagai nagusiak dira, baita metabolismoaren eta zelula-seinaleztapenaren eragile garrantzitsuak ere. Lipido horiek glizerol kate bat dute oinarri moduan, eta bi FA sn-1 eta sn-2 posizioetan lotuta. Maiz, GPL-ek sn-1-en FA ase bat eta sn-2n FA asegabe bat izaten dituzte. Glizerolaren hirugarren karbonoan fosfato bat dute, eta jarraian buru polar bat. Hortaz, molekula anfipatikoak dira, buru polar bat eta bi kate hidrokarbonatu hidrofobikoak baitituzte. Buru polar horien izaeraren arabera GPL-ak azpimota ezberdinetan banatzen dira. Bestalde, buru polarraren, karbono katearen luzera eta insaturazio kopuruaren arabera, lipidoen funtzio biologikoak aldatzen dira, eta ondorioz, lipido horiek eratzen dituzten mintzen propietate fisiko-kimikoak ere aldatzen dira.

-Azido fosfatidikoa (PA) edo fosfatidatoa beste glizerofosfolipido eta triazilglizerol batzuen sintesirako bitartekaria da, eta, beraz, kopuru txikietan agertzen da ehunetan. Glizerol molekula batek osatzen du, zeinak sn-1 eta sn-2-tan bi FA esterifikatuta dituen, eta hirugarren karbonoan, azido fosforiko bat duen. Beraz, PA-ren talde polarra hidrogeno bat da. Gutxienez lau bide desberdinetatik sor daiteke PA. Bide nagusia erretikulu endoplasmikoan edo mitokondrietan gertatzen da, GPAT entzimak (glizerol-3-fosfato aziltransferasa) glukosaren katabolismoan sortzen den sn-3-glizerol-3-fosfatoan bi azilazio sekuentzial egiten ditu. PA-ren beste bide sintetiko bat GPL ezberdinen hidrolisiaren ondoren ematen da. Adibidez, PLD1 eta PLD2 entzimek kontrolatzen duten bidean, fosfatidilkolina (PC) baten hidrolisiaren ondorioz PA eta buru polar aske bat ekoizten da[51,52].

Beste lipido batzuen aitzindaria izateaz gain, frogatu da seinaleztapen-molekula gisa ere jarduten duela. Izan ere, PAren presentziak eragina du zelulen hazkuntzan, mugikortasunean eta biziraupenean[51,52].

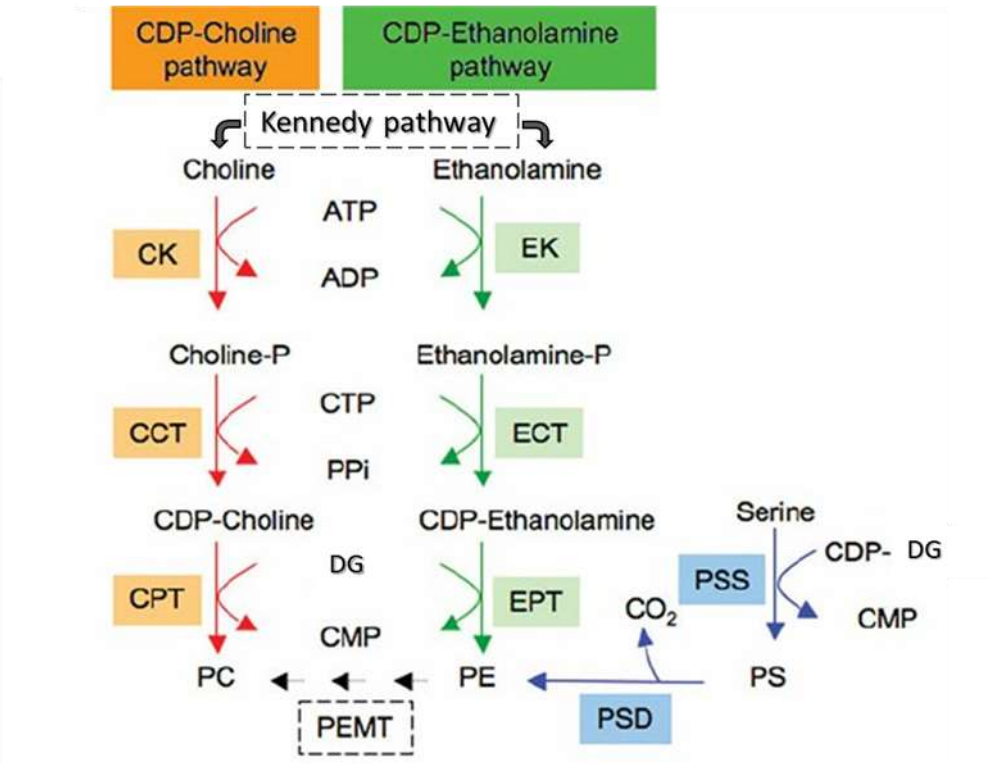
- Fosfatifilkolinek (PC) kolina molekula bat dute buru polar moduan. Maiz, PC molekulek azido palmitikoa (16:0) edo azido estearikoa (18:0) FA-k dituzte sn-1 kokapenean, eta GA asegabe bat sn-2-an, esaterako, azido oleikoa (18:1), azido linoleikoa (18:2) edo azido linolenoikoa (18:3)[52,53].
  Kolina taldea funtsezko elikagaia da gizakientzat, batez ere haurdun dauden emakumeentzat eta jaioberrientzat, gorputzak ezin baitu sintetizatu eta dietatik hartu

behar da. Giza gorputzak kolina behar du fosfatidilkolina, kolina eter lipidoak eta esfingomielinak sintetizatzeko; lipido klase horiek guztiak zelula-mintzen egituraren funtsezko osagaiak dira. Kolinaren xurgapena kolina-garraiatzaileek arautzen dute, eta hori batez ere PC-ak sintetizatzeko erabiltzen da. Glizerofosfolipido horiek oso ugariak dira zelula mintzaren lipido geruza bikoitzean, bereziki kanpoko xaflan, mintzetako glizerofosfolipido guztien %50 inguru osatzen dutelarik[54]. PC-en egituran FA asegabeak egoteak jariakortasuna ematen die zelula-mintzei. Gainera, hain garrantzitsua den azetilkolina neurotransmisorea sortzeko ere beharrezkoa da kolina.

Bide gehigarriak dauden arren, PC sintesi-bide nagusia Kennedy-ren bidea da **(12. Irudia)**. Horretan, zelularen barnealdera garraiatzen den kolina taldea, kolina kinasaren (CK) bitartez fosforilatzen da zitoplasman, eta CTP:fosfokolina citidiltransferasaren (CCT) bidez zitidina trifosfatoa (CTP) gehitzen zaio molekula aktibatuz. Sortutako CDP-kolina erretikulu endoplasmikora garraiatzen da. Bertan, CDP-kolinak lipido aingura gisa erabiltzen den diglizerido batekin erreakzionatzen du PC bat sortuz. Gibelean, PC-ak ekoizteko beste sintesi-ibilbide bat gertatzen da nagusiki, non PE metiltransferasak (PEMT) fosfatidiletanolamina (PE) molekularen hiru metilazio sekuentzial katalizatzen dituen, PC bat sortuz[55] **(12. Irudia)**. PC-en sintesiaren ondoren Lands zikloa izeneko birmoldaketa-prozesua gertatzen da lipido horien egitura aldatuz **(14. Irudia)**. Prozesu metaboliko hori izugarri garrantzitsua da GPL guztietan. Izan ere sintesiaren ondoren FA-en kateak askatzen dira A_2 fosfolipasa entzimaren eraginez, eta FA berriak eransten zaizkio berazilazioaren bitartez.

Bestalde, PC-ek hainbat prozesu katabolikoetan hartzen dute parte. Fosfolipasa entzimek GPL ezberdinen hidrolizazioa arautzen dute, geroago 3. atalean (232. orrian) sakonago azalduko den moduan. D fosfolipasaren familiako entzimek hidrolizatzen dituzte PC-ak, PA eta kolina askatuz. Kolina molekula hori askotan PC berriak ekoizteko erabiltzen da. Gainera, C fosfolipasaren bidez, DG eta fosfokolina sortzen dira, eta A fosfolipasa familiako entzimek (PLA_1 eta PLA_2) gantz-azido aske bat eta liso-fosfatidilkolina sortzen dute. Ibilbide katalitiko horietan sortutako azpiproduktu guztiek seinaleztapen-molekula garrantzitsuak dira.

Deigarria da PC-ak beste lipido batzuen sintesiaren aitzindariak direla, esfingomielinak, plaketak aktibatzeko faktorea (PAF) eta PE-k barne. Horiek guztiek egitura eta seinaleztapen-funtzio garrantzitsuak betetzen dituzte[53,55].



Irudia 12. De novo sintesi-bideak PC, PE eta PS molekulak sortzeko. Epand *et al*.-etik hartuta. Laburdurak: CK –kolina kinasa, CCT – CTP:fosfokolina citidiltransferasa; CPT –kolina fosfotransferasa; EK – etanolamina kinasa; ECT –CTP:fosfoetanolamina citidiltransferasa; EPT –etanolamina fosfotransferasa; PEMT –PE metiltransferasa; PSS –fosfatidilserina sintasa; PSD –fosfatidilserina descarboxilasa; ATP – adenosina trifosfato; ADP –adenosina difosfato; CTP – citidina trifosfato; PPi –pirofosfato; DG –diglizeridzo CMP –citidina monofosfato; CDP-DG – citidina difosfato diglizeridoa.

- Fosfatidiletanolaminek (PE) etanolamina bat dute talde polar gisa, eta ugaztunen artean bigarren GPL ohikoenak dira. PE horiek giza gorputzeko zelula bakoitzean daude, GPL guztien %25 inguru osatzen dutelarik; nerbio-ehunean, berriz, GPL guztien %45 ere izan daitezke. Gainera, bere ugaritasuna organulu batetik bestera aldakorra da, mitokondrioetan kopuru handiagoak metatuz. PC-ak bezala, normalean, azido palmitikoa (16:0) edo azido estearikoa (18:0) dute sn-1 kokapenean, eta FA asegabe luzeago bat (20-22 karbono) sn-2 kokapenean. PC-ekin batera, zelula-mintzen osagai nagusiak dira, eta lipido geruza bikoitzaren alde zitoplasmatikoan aurki daitezke nagusiki. Hala ere, mitokondria-mintzaren barneko nahiz kanpoko xafletan agertzen dira, eta funtzio garrantzitsuak betetzen dituzte bertan. Bereziki, proteinen mugimendua mintzetan zehar eta mintzen fusioa errazten dute. Bestalde, gibelean sintetizatutako PC molekulen %35-aren aitzindariak dira. Garunean, anandamida estekatzaile kanabinoidearen sintesiaren aitzindariak dira. Gainera, mitosirako garrantzitsuak dira, Golgiren mintz mitotikoen fusioa kontrolatzen baitute. Autofagian ere garrantzitsuak dira. Beraz, garrantzitsuak dira ez bakarrik beren funtzio estrukturalagatik, baita funtsezko prozesu zelular askotan parte hartzen dutelako ere. Gainera, hazkuntza-faktore gisa jarduten dute zelula-kultiboetan, eta serum baxuko hazkuntza-baldintzek sortutako apoptosia gainditzen laguntzen dutela frogatu da[26,56,57]. PE-k hainbat bide ezberdinetatik sintetiza daitezke. PC-ek bezala, Kennedy bidea dute sintesi-bide nagusitzat. Hala ere, PE-tan etanolamina da substratua eta bere fosforilazioa etanolamina kinasaren (EK) bidez egiten da. Sintesi bidearen bigarren

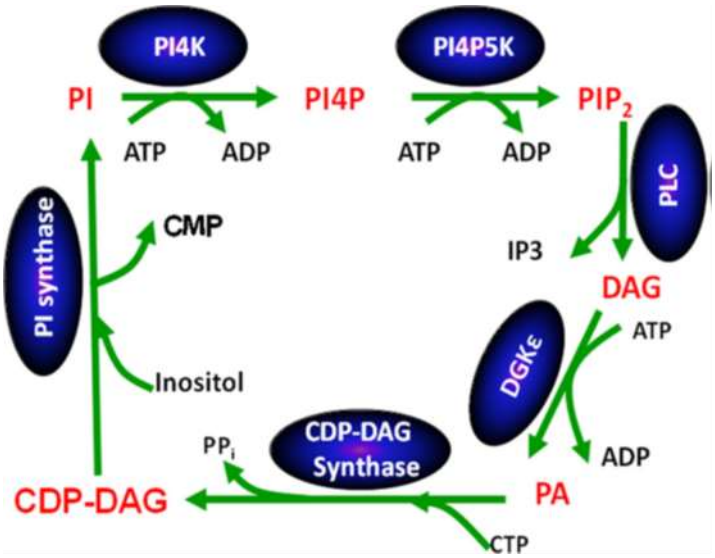
urratsean, CTP fosfoetanolaminarekin kondentsatzen da ECT entzimaren eraginez (CTP: fosfoetanolamina citidiltransferasa), eta CDP-etanolamina sortzen da. Azken urratsean, EPT-k (etanolamina fosfotransferasa) CDP-etanolaminaren eta lipido-aingura baten (normalean DG) arteko erreakzioa katalizatzen du, PE sortuz. *De novo* sintetizatutako PE-k, nagusiki, sn-2 kokapenean azilo-kate monoinsaturatu edo diinsaturatuak dituzte, 18:2 kateak kasu[26,55]. PE-en bigarren iturria mitokondrioak dira, non fosfatidilserina deskarboxilasak (PSD) PS molekula bat deskarboxilatzen duen PE bat sortuz **(12. Irudia)**. PE-ak sintetizatzeko beste bide minoritario bat erretikulu endoplasmatikoan ematen dena da. Hau kaltzioaren mendeko base-truke erreakzio bat da, non PS baten serina etanolamina baten ordez aldatzen den[56,57]. PC-k bezala, PE molekulen sintesiaren ondoren, molekula hauek Lands zikloan birmoldatzen dira **(14. Irudia)**.
Ugaztunek, ordea, ezin dute etanolamina molekula sintetizatu, eta, beraz, dietatik etanolamina aske gisa eskuratzen da, edo jada gorputzean dauden beste PE-etatik lortzen da. Fosfodiesterasek PE-ak hidrolizatzen dituzte, etanolamina eta glizerol askeak ekoizteko. Beste etanolamina iturri bat esfingosina fosfatoaren degradazioa da, esfingosina fosfato liasaren bitartez, eta anandamida endokannabinoidearen lisia gantz-azido amina hidrolasaren bidez[56].

- Fosfatidilserinek (PS) serina-talde bat dute eta GPL guztien %3-15 ordezkatzen dute. Garuna eta erretina, ordea, PS-ekin aberastuta daude. Organuluen artean ere, PS kontzentrazio ezberdinak daude, mintz plasmatikoan ugariak baitira, bereziki barne xaflan, eta aldiz, mitokondrioen barne-mintzean, urriak[58].
  Ugaztunetan, lipido horien sintesia kaltzioaren menpeko buru polar baten (kolina edo etanolamina) ordezkapen baten bidez gauzatzen da. Zeluletan dauden PC edo PE molekulen buru polarra serinarekin ordezkatzen da. Aldiz, PS-k deskarboxilatu daitezke mitokondrietan PE-k ekoizten direlarik.
  PS-en funtzio nagusiak hauek dira: PE-aren aitzindariak izatea mitokondrioetan, proteina batzuk fagosometara zuzentzea eta entzima batzuen jarduera katalitikoa aldatzea, V anexina eta C proteina kinasa kasu. PS-en ezinbesteko papera egiaztatzeko, publikatutako beste lan batean, arratoietan PS sintesia kaltetzeko mutazio bat sortu zuten, eta arratoi horiek ez zuten bizirik iraun. Izan ere, PS-ek odolaren koagulazioan eta apoptosian parte hartzen dute[57–59].

- Fosfatidilinositolek (PI) inositol molekula bat dute buru polar bezala, hexalkohol zikliko bat dena. Funtsean, azido estearikoa (18:0) dute sn-1-en eta azido arakidonikoa (20:4) sn-2-n. Zelulen mintzen kanpoko xaflaren osagai estrukturalak dira, GPL guztien %10 inguru osatzen dutenak. Zelula-mintzen kanpoko azalean proteinak ainguratzen laguntzen dute, zelulen seinaleztapenean oso paper garrantzitsua betez[60].
  PI-n *de novo* biosintesia PI-zikloan egiten da **(13. Irudia),** PC eta PE-en Kennedy bidearen antzekoa. Lehenik, erretikulu endopla smikoan, CDP-DG inositolarekin elkartzen da, fosfatidilinositol sintasak katalizatua. Gero, sortutako PI molekula birmoldatu egin daiteke Lands zikloaren bidez. Bertan, PI-ak birmoldatzeko azetiltransferasa espezifiko bat dago, LPIAT (liso-PI aziltransferasa) deritzon entzima. PI-ak azido arakidonikoaren iturri nagusia dira, eikosanoideen biosintesirako behar dena[52,60,61].
  PI zikloaren barruan, inositol eraztuna modu itzulgarrian fosforilatu daiteke 3, 4 eta/edo 5 posizioetan, 7 fosfoinositido molekula desberdin (PPIn) sortuz. Hauek fosfatidilinositol fosfatoa (PIP), fosfatidilinositol bifosfatoa (PIP_2) edo fosfatidilinositol trifosfatoa (PIP_3) izan daitezke, seinaleztapen-molekula oso garrantzitsuak direnak. Zelulen mintzetan

fosfolipido guztien %1 baino ez badira ere, bigarren mezulariak izan daitezke edo mintzaren proteina desberdinen jarduera eta ainguraketa modulatu dezakete. Era horretan oinarrizko hainbat zelula-prozesutan aktiboki parte hartzen dute, hala nola seinaleen transdukzioan, mintzaren dinamikan, zitoeskeletoaren berrantolaketan, mintzaren eta besikulen trafikoan[52,62].

Bestalde, molekula horien fosforilazioa asko ikertu da, PI3K entzimak (fosfatidilinositol 3-kinasa) bultzatzen baitu, eta entzima hori minbizi askotan mutatuta dagoela ikusi da. Gainera, PI3Kren desfosforilazioaren erantzule den PTEN fosfatasa lipidikoa hainbat minbizi motatan ere maiz mutatuta agertzen da. PTEN-en ematen den mutazio horrek bere inaktibazioa eragiten du, eta beraz, ez du PI3K desfosforilatzen. Mutazio horiek PIP_3 molekulen adierazpena areagotu egiten dute, eta ezaugarri horrek lotura estua du gaitasun metastatiko altuarekin. Beraz, PI3K zein PTEN minbiziari aurre egiteko itu farmakologiko oparoak izan daitezke[52,62,63].



Irudia 13. PI zikloa. Borobil urdinetan bide bakoitza katalizatzen duten entzimak daude. Gorriz sortzen diren bitartekari lipidikoak daude[61].

- Fosfatidilglizerola (PG) glizerol-3-fosfatozko egitura du, bi FA esterifikatuta dituena sn-1 eta sn-2-an, eta beste glizerol molekula bat buru polar bezala. Normalean mitokondrioaren barne mintzean kokatzen da. Mintzetan kopuru baxuan agertzen den arren (PL guztien %1), bere funtzio nagusia kardiolipina mitokondrialen aitzindaria izatea da.

  Azpiklasearen *de novo* biosintesia CDP-DAG eta glizerol-3-fosfatoaren kondentsazioaren bidez gauzatzen da, fosfatidilglizerolfosfato sintasak katalizatua, fosfatidilglizerol-fosfatoa sortuz. Gero, bitartekaria desfosforilatzen da fosfatidilglizerolfosfatoa fosfatasarekin PG fosfolipidoa sortuz. Gainerako GPL-etan bezala, PG-en FA-k Lands zikloan birmoldatu daitezke. Zehazki, PG-ren aziltransferasa espezifiko bat aurkitu da (LPGAT1). Gainontzeko GPL-ak ez bezala, askotan FA insaturatu bat dute sn-1 kokapenean[64,65].

  PG-ek ugaztunen zeluletan duten ugaritasun txikia dela eta, oso gutxi ezagutzen da euren funtzio fisiologiko eta patologikoei buruz.

- Kardiolipinek egitura dimeriko esklusiboa dute, glizerolezko molekula zentral batez osatuta daude, zeinak bi azido fosfatidiko dituen lotuta. Beraz, lau FA dituzte, normalean kate monoinsaturatu edo diinsaturatuak direnak 16-18 karbonorekin. Gainera, aztertu diren kardiolipina-egituren ia erdia simetrikoak dira, lau azilo-kateen barruan karbonoen eta lotura bikoitzen banaketa homogeneoa baita. Nagusiki mitokondrioen barne-mintzean aurkitzen dira, egitura horretako GPL guztien %20 izanik[66]. Bertan, mintza fusionatzeko eta elektroien garraio-kateko entzima batzuen funtzio entzimatikoa mantentzeko garrantzitsuak dira. Horrela, zelulen energia produkzioan eraginez eta fosforilazio oxidatiboan protoiak harrapatuz[68,69].

- Eter fosfolipidoek alkilo edo alkenilo/binilo lotura bat dute glizerolaren eta gantz-alkohol baten artean sn-1 kokapenean. Eter lipidoak, GPL azpiklase guztietan ematen diren arren, kolina eta etanolamina eter lipidoak dira ohikoenak. Konkretuki, plasmalogenoak ugarienak dira, eta lotura bikoitz bat dute eter loturaren ondoan, horrela binilo-eter lotura bat eratuz. Normalean, sn-1 kokapenean agertzen diren gantz-alkoholak 16:0, 18:0 eta 18:1 izaten dira. Aldiz, sn2-an FA poliinsaturatuak kontzentratzen dira, normalean azido dokosahexaenoikoa (22:6) edo azido arakidonikoa (20:4). Alkilo-eter loturak, normalean, kolina duten eter fosfolipidoetan aurkitzen dira, eta binilozko eter loturak, ordea, nabarmenagoak dira etanolamina duten plasmalogenoetan[70,71].

  Bere presentzia oso aldakorra da, ehunaren edo organoaren araberakoa baita; glizerofosfolipido guztien %20 inguru osatzen dute, bere ugaritasuna handituz bihotzean, nerbio-ehunean eta hantura-zeluletan. Zelula barruan, mintz-plasmatikoan, mintz-nuklearrean, erretikulu endoplasmatikoko mintzean eta mintz-mitokondrialean agertzen dira batez ere. Lipido horien funtzio nagusiak nukleoko eta mitokondrioetako mintzetan euskarri izatea da, baita besikula zelularren eraketa, mintzen fusioa eta ioien garraioan parte hartzea ere. Mintz biologikoetan plasmalogenoak egoteak bigeruza lipidikoa osatzen duten fosfolipidoen paketatze estuagoa eragiten du, eta horrek mintzaren gogortasuna areagotu eta jariakortasuna gutxitzen du. Gainera, bigeruzetako baltsa lipidikoetan aberastuta aurkitu dira, eta lipido horien metaketa, esfingolipidoekin eta kolesterolarekin batera, mintzeko lipido baltsen egonkortasun handiagoarekin lotu da. Izan ere, hainbat ikerketek agerian utzi dute mintzetan plasmalogenoen presentzia blokeatzeak estres oxidatiboa eta apoptosi zelularra dakarrela, lipido horiek mintzaren homeostasian duten garrantzia nabarmenduz. Gainera, seinaleztapen-funtzio garrantzitsuak eta antioxidatzaileak deuseztazeko propietateak ere badituzte. Izan ere, binil-eter lotura gai da oxigeno espezie erreaktiboak harrapatzeko eta hain sentikorrak diren PUFA espezieen oxidazioa saihesteko. Gainera, oxidazio-azpiproduktu gutxiago sortzen dituzte, beste fosfolipido batzuekin alderatuta[70,72–74].

  Eter lipidoak bide biosintetiko ezohiko eta konplexuetatik sintetizatzen dira, peroxisometan hasi eta erretikulu endoplasmikoan amaitzen direnak. Peroxisomak organulu metaboliko esanguratsuak dira, prozesu metaboliko oso garrantzitsuetan parte hartzen baitute, horien artean FA adarkatuen eta kate luzekoen oxidazioa, eter fosfolipidoen sintesia, azido biliarren sintesia, aminoazidoen katabolismoa, poliaminen oxidazioa eta pentosa fosfatoen bideko alde oxidatiboa (PPP)[72,74,75].

  Eter fosfolipidoen sintesirako, lehenik eta behin, peroxisomaren barnean, dihidroxiazetona fosfato (DHAP) molekula kate luzeko azil-CoA batekin esterifikatzen

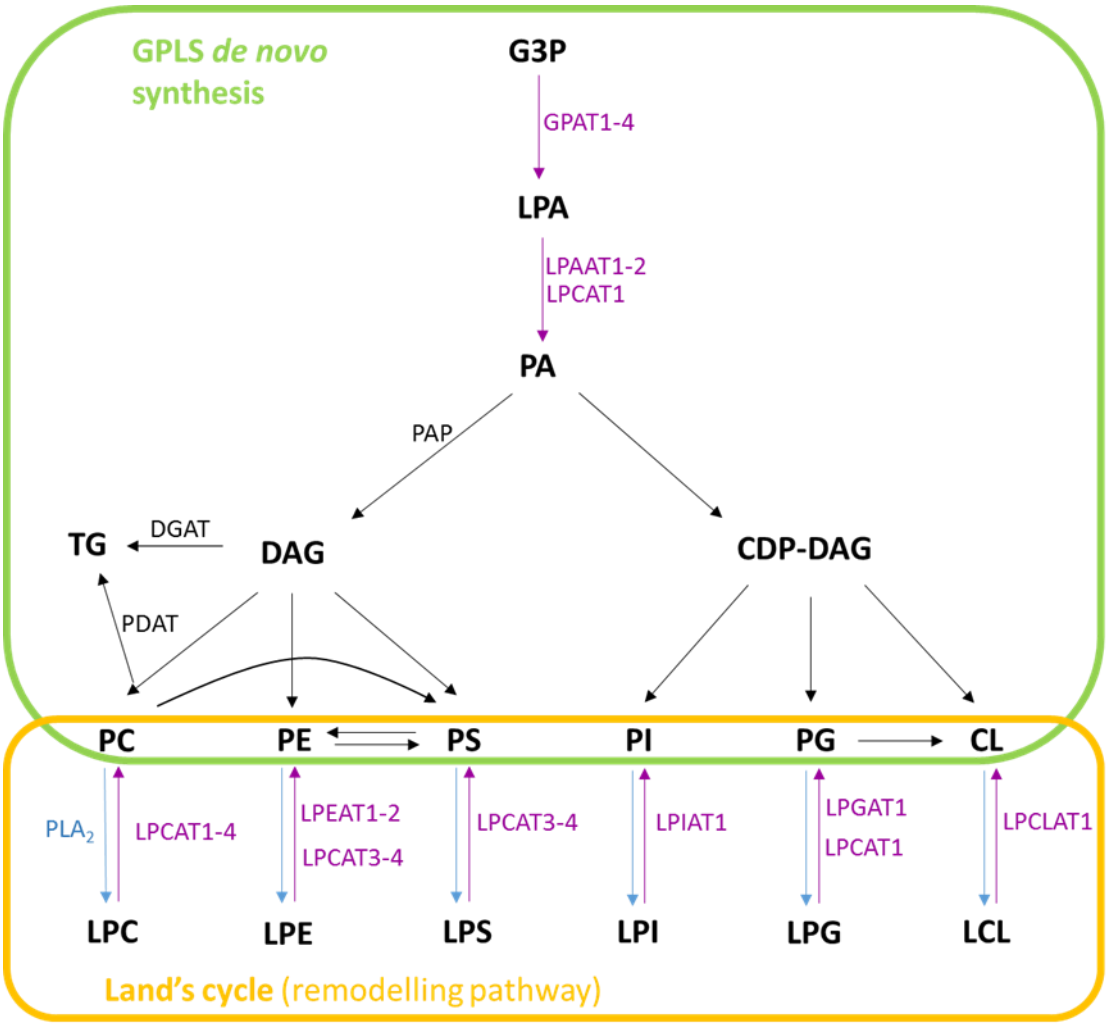
da, aziltransferasa dihidroxiazetonafosfato (DHAPAT) entzimaren eraginez. Gero, eter lotura, alkilo-DHAP sintasa (ADAPS) bidez sartzen da, azil-DHAP molekularen azil katea kate luzeko gantz-alkohol batekin ordezkatzen da. Gainerako erreakzio katalitikoak erretikulu endoplasmikoan gertatzen dira, eta beraz, 1-alkilo-DHAP bitartekaria organulu horretara garraiatzen da. Erretikulu endoplasmatikoan ematen den lehenengo erreakzioa zetona taldearen erredukzioa da, glizerol molekula bat sortzeko. Gainera, sn-2 kokapenean azilo-talde bat sartzen da, 1-alkil-2-azil-glizero-3-fosfato bat sortuz. Ondoren, fosfohidrolasa batek fosfato taldea kanporatzen du, eta entzima ezberdinen jardueraren ondoren, bitartekaria etanolamina edo kolina eter fosfolipido bihurtzen da, Kennedyren ibilbidearen antzeko prozesu batean. PC-ak bezala, PC-eterrak PE-eterren metilazioaren bidez ere sintetizatu daitezke, erretikulu endoplasmatikoan eta mitokondrioetan dagoen PEMT entzimaren bidez. Azkenik, ekoiztutako lipidoak erretikulu endoplasmatikotik beste organuluetara eta mintz plasmatikora garraiatzen dira, etanolamina-plasmalogenoak mintzen barneko xaflan banatzen direlarik, eta kolina eter-fosfolipidoak aldiz, kanpoaldean[65,71,72].

Eter lipidoak ere fosfolipasen bidez birmoldatzen dira, eter lipidoen PLA_2 isoforma espezifiko bat baitago. Horrela, eter lipidoak seinaleztapen lipido garrantzitsutzat hartzen dira, oro har AA, azido dokosapentaenoikoa edo azido docosahexaenoikoa (DHA) baitute sn-2-an, eta horiek PLA_2-ren jarduerarekin askatzen dira[70–72].

Glizerofosfolipidoen azpimota ezberdinak *de novo* bide espezifikoen bidez sintetizatzen dira, **14. Irudian** laburbiltzen direnak. *De novo* biosintesi bide horiek errretikulu endoplasmatikoan, peroxisometan eta mitokondrioetan ematen dira, nahiz eta birmoldaketaren azken urratsak errretikulu endoplasmatikoan edo mitokondrioetan eman daitezkeen. Fosforilatzen den glizerol molekula batetik abiatuta, azido liso-fosfatidiko (LPA) bat sortzen da, glizerol-3-fosfato molekula eta FA bat, normalean asea, elkartuz, glizerol-3-fosfato aziltransferasa (GPAT) entzimaren bitartez. Gero, bigarren FA bat esterifikatzen da LPAn, eta PA bat sortzen da azil-CoA liso-PA aziltransferasen bidez (LPAAT). Eratutako PA, metabolizatu egiten da glizerol deribatu ezberdinak eratzeko. Bat, diazilglizerola (DAG edo DG), PA-ren desfosforilazioaren ondorioz sortzen da, fosfatasa azido fosfatidikoaren bidez (PAP). DAG hori bitartekaria da TG -en sintesirako, DAG-ari azilo-kate bat gehitzen baitzaio diazilglizerol aziltranferasaren (DGAT) bidez edo fosfolipido:diazilglizerol aziltransferasak (PDAT) azilo-kate bat transferitu dezake PC molekula batetik DAG batera. Gainera, PC, PE eta PS fosfolipidoak ere DAG-etik abiatuta ekoizten dira. PS-a ere sor daiteke PC eta PE-ak eraldatuz. Glizerolaren beste deribatua DAG-zitidinadifosfatoa (CDP-DAG) metabolizatuz PI-ak, PG-ak, CL-ak edo PS-ak eratzen dira.

Zelula-mintzen propietate biofisikoak egitura horietan dauden fosfolipidoen ezaugarrien araberakoak dira. Ehun desberdinetako lipido edukia aldakorra da, baita zelula beraren barruko organuluen artekoa. Horrek garrantzi handia du konpartimentu bakoitzaren funtzio espezifikoetan islatzen baita. Zehazki, lipidoen aniztasuna buru-polar ezberdinek, azilo-kateen luzerak eta insaturazio desberdinek ematen dute eta ondorioz, geruza bikoitzaren jariakortasuna, iragazkortasuna, egonkortasuna, kurbadura eta azpidomeinuen arkitektura zehazten dute. Hori guztia besikula-trafikoan, seinaleen transdukzioan eta zelulen garraio molekularrean islatzen da. Beraz, mintzen fosfolipido edukiak zuzenean eragiten die prozesu zelular horiei guztiei. *De novo* sintetizatutako GPL-ak birmoldatzeko prozesua Lands zikloa da. *De novo* sintesi-bideetako entzimek FA konkretuetarako substratu-espezifikotasun gutxi dutenez, sortutako GPL-ek mintzek behar duten funtzio jakinetarako azilo-kate egokiak ez edukitzea eragin dezakete. Beraz, glizerofosfolipidoek euren FA-en kateak aldatzen dituzte

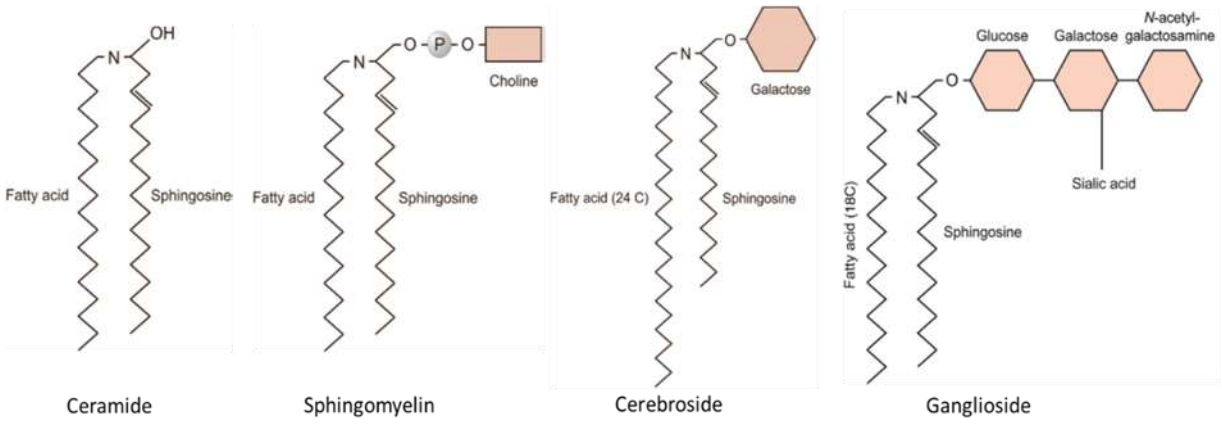
desazilazio eta birazilazio erreakzio batzuen bidez. Uste denez, sn-1 kokapenean agertzen den FA asea, orokorrean, *de novo* sintesitik eratortzen da; sn-2 kokapenean ikusten den FA asegabea aldiz, birmoldaketa prozesu horretan sartzen da. Horretarako, A2 fosfolipasak (PLA2) GPL-aren sn-2 kokapenean dagoen azilo-katea mozten du. Beraz, seinaleztapen-molekula garrantzitsuak askatzen dira prozesu honen eraginez, liso-fosfolipidoak eta gantz-azido askeak askatzen baitira, hain garrantzitsua den azido arakidonikoa barne (20:4). Azken hori eikosanoide bihur daiteke, prozesu fisiologiko eta patologiko askotan inplikatua dagoena, tartean hantura, erantzun immunea, loaren erregulazioa eta minaren pertzepzioa. Gero, sortutako liso-GPL-a birazilatu daiteke, bestelako gantz-azido bat erantsiz eta azil-CoA:lisofosfolipido aziltransferasa (LPLAT) espezifiko baten bidez fosfolipido berri bat eratuz. Beraz, prozesu horrek oxidatu diren gantz-azidoak ordezka ditzake mintzen barruan azilo-kateen eduki anitza eta asimetrikoa sortuz, azkenik mintzek behar bezala funtziona dezaten[76–79].



Irudia 14. Glizerofosfolipidoen biosintesia. *De novo* sintesi bide ezberdinen eta Lands zikloaren konbinazioak fosfolipidoen dibertsitatea sortzen dute. Gezi urdinek PLA_2 adierazten dute eta gezi moreek aziltransferasak. G3P, glizerol-3-fosfato; GPAT, glizerol-3-fosfato aziltransferasa; LPA, liso-PA; LPAAT, liso-PA aziltransferasa; LPCAT, liso-PC aziltransferasa; CDG-DAG, zitidina difosfato-DAG; LPE, liso-PE; LPC, liso-PC; LPS, liso-PS; LPI, liso-PI; LPG, liso-PG; LCL, liso-CL; LPEAT, liso-PE aziltransferasa; LPIAT, liso-PI aziltransferasa; LPGAT, liso-PG aziltransferasa; LPCLAT, liso-CL aziltransferasa.

Esfingolipidoak (SPL) egitura ezaugarri komun bat partekatzen duten lipido konplexuen familia bat da. Hau da, base esfingoide bat dute oinarri gisa, amino-alkohol alifatiko ezberdinez osatua dagoena. Ohikoena esfingosina da, 18 karbonoko luzera duen kate alifatiko bat, 4. posizioan lotura bikoitzarekin, 1. eta 3. posizioetan hidroxilo taldeak, eta amina talde bat 2. posizioan duenak. Base esfingoideak *de novo* sintetizatzen dira serina aminoazido baten eta kate-luzera eta insaturazio kopuru ezberdineko gantz-azil-CoA baten kondentsazioaren ondorioz. Esfingosina da base esfingoide arruntena, eta serina eta azido palmitikoaren kondentsazioaren ondorioz sortzen da. Bestalde, base esfingoidera lotzen diren gantz-azidoak, lipido familia honetan ez dira GPL-tara lotzen diren antzekoak. Kasu honetan, gehienez 26 karbonoko kate luzeak izan daitezke eta karbono atomo kopuru bikoitiak edo bakoitiak izan ditzakete. Hala ere, epidermisean aurkitu daitezkeen esfingolipidoetan 28 eta 36 karbono arteko FA-k egon daitezke. Bestalde, FA poliinsaturatuak oso gutxitan aurkitzen dira esfingolipidoetan[80].

Hainbat lipido bioaktibo daude esfingolipidoen familian: zeramidak, esfingosina 1-fosfatoa (S1P), esfingosina eta glikolipidoak. Kartzinogenesiarekin lotura estua duten hainbat prozesu zelularretan parte hartzen dute, hantura, seneszentzia, apoptosia, angiogenesia, zelulen hazkuntza, biziraupena, migrazioa eta bereizketa arautzen baitituzte[63].



Irudia 15. Esfingolipidoen egitura orokorraren irudikapen eskematikoa[54].

- Zeramidak (Cer) gantz azido bat eta base esfingoide bat amida lotura baten bitartez lotzen direnean sortzen dira. Molekula horien azilo-kateak oso ezberdinak izan daitezke euren jatorri biologikoaren arabera, gehienetan azilo-kate aseak edo monoinsaturatuak diren arren. Larruazala ez den beste ehunenetan presentzia urria izan arren, berez funtzio biologiko garrantzitsuak dituzte mezulari lipidiko garrantzitsuak baitira. Gainera, zeramidak esfingolipido konplexuagoen aitzindariak izan daitezke, eta funtzio ugari betetzen dituzte zeluletan[81]. Esaterako, zeramidek funtzio estrukturalak betetzen dituzte mintzetan, lipidozko geruza bikoitzen iragazkortasuna aldatzen baitute mintzetan dauden kanal ionikoekin interakzioan. Zeramidak mintzen osagai minoritarioak badira ere, hainbat seinaleztapen-bideetan parte hartzen duten lipido baltsetan pilatzen dira, normalean entzima katabolikoak aktibatuz eta prozesu anabolikoak motelduz. Beraz, eragile garrantzitsuak dira eraldaketa zelularra, bereizketa, hazkuntza, migrazioa, heriotza zelularra, autofagia, seneszentzia eta apoptosia arautzeko. Izan ere, agente kimioterapeutiko askoren eta hainbat erradazio

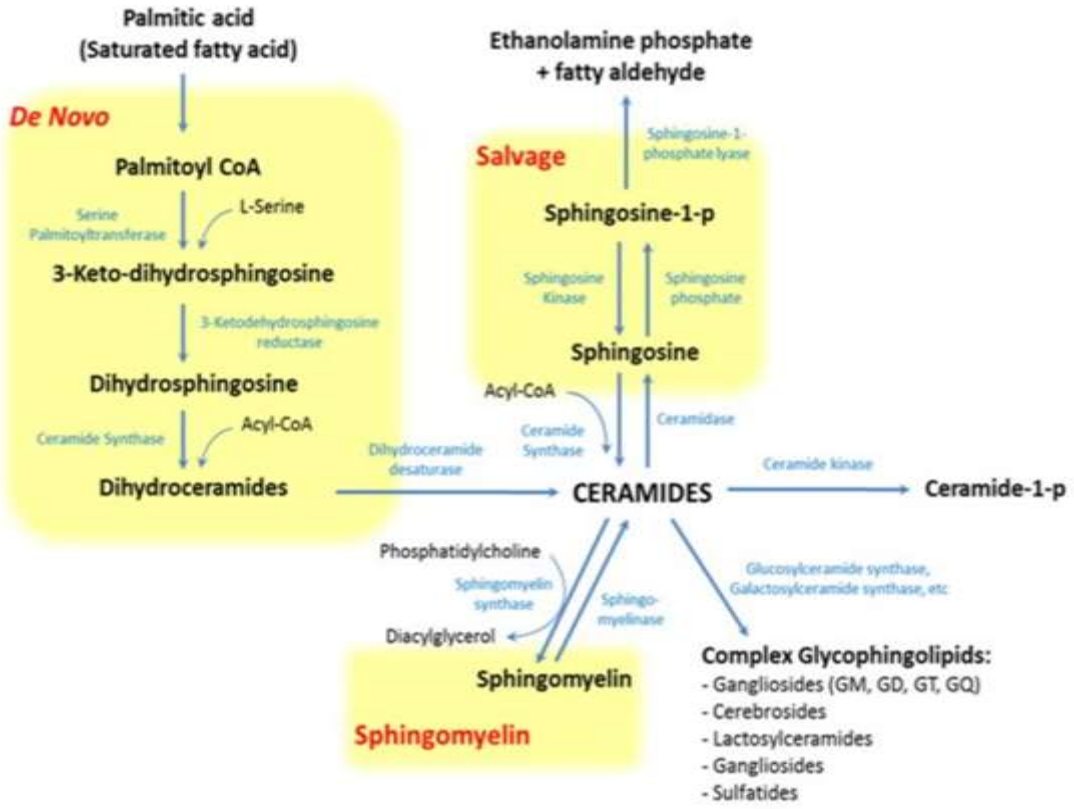
bidezko tratamenduren ekintza-mekanismoak zeramida edukiaren igoerak eragiten duen heriotza zelularrean oinarritzen dira[81–83].

Deigarria da zeramida pilaketa handia dagoela larruazalaren geruza korneoan, zehazki egitura honetan dauden lipido guztien %50 baitira. Bertan, zeramidak molekula aske zein egiturako osagaiak diren proteinetan esterifikatuak agertzen dira. Gainera, karbono-kate luzeagoak dituztenez, ezaugarri desberdinak dituzte. Horrela, larruazalari irazkortasun txikiagoa ematen diote, larruazalak duen babes-funtzioan lagunduz[82].

Zeramiden biosintesia konplexua da eta hainbat bidetatik eman daiteke **(16. Irudia)**. Lehenik, erretikulu endoplasmikoan *de novo* sintetizatu daitezke. Serina eta palmitoil-CoA baten kondentsazioarekin hasten da, eta, ondoren, CoA askatzen da palmitoiltransferasa serinagatik (SPT), 3-zeto-dihidrosfingosina sortuz. Jarraian, hori erreduzitu egiten da esfinganina (dihidrosfingosina) eratzeko 3-zetosfinganina-erreduktasarekin. Esfinganina kate oso luzea duen FA batekin kondentsatzen da, dihidrozeramida bat sortuz dihidrozeramida sintasaren eraginez (CerS). CerS-en sei isoforma ezberdin aurkitu dira, sintetizatzen duten zeramidaren karbono katearen luzeraren arabera. Adibidez, CerS3-k kate oso luzeko (28-32 karbono) FA poliinsaturatuekin sortzen diren zeramiden sintesian parte hartzen du; horiek larruazalean asko adierazten dira[84]. Bukatzeko, lotura bikoitz bat sartzen da base esfingoidearen 4. kokapenean dihidrozeramida desaturasaren bidez. Bitarteko gisa erabiltzen diren zeramida gehienak erretikulu endoplasmikoaren alde zitoplasmatikoan sintetizatzen badira ere, SPL konplexuagoen ekoizpena Golgi aparatuan gauzatzen da. Beraz, SPL konplexuagoen sintesian erabiliko diren zeramidak, Golgi aparatura garraiatzen dira proteina zitoplasmatiko giltzarri baten bitartez, zeramida-garraiatzaileak (CERT) hain zuzen ere[82,85,86].

Zeramiden biosintesirako beste bide bat SPL konplexuen katabolismoa da, lisosomen barnean ematen dena eta salbazio bidea deritzona. Ibilbide horrek *de novo* sintesiak baino askoz azkarrago sortzen ditu zeramidak. Beraz, seinaleztapen azkarreko bideetarako garrantzi handikoa da. Bertan, S1P desfosforilatzen da esfingosina kinasa entzimaren eraginez, eta sortutako esfingosinari FA bat heltzen zaio zeramida sintasen bidez, zeramida molekula bat sortuz[86]. Zeramiden sintesirako hirugarren bidea SM zikloa da, non esfingomielinasek (SMasak) esfingomielina (SM) katabolizatzen duten, zeramida eta fosfokolonia bat sortuz. Hiru SMasa mota daude pH-aren menpeko jarduera optimoaren arabera: SMasa azidoak, neutroak eta alkalinoak[87]. Bestalde, glikoesfingolipidoak ere hidrolizatu daitezke glikosidasen bitartez zeramidak sortzeko. Hala ere, bide hau ez da oso garrantzitsua animalia ehunetan.

Gero, zeramidasek askatutako zeramidak metabolizatu ditzakete, esfingosina base bat eta FA aske bat sortuz. Izan ere, bost zeramidasa desberdin daude, lokalizazio azpizelular desberdinekin eta FA-ekiko espezifikotasun desberdinarekin, eta horrek zelula- eta seinaleztapen-gertaera ezberdinei eragiten die. Bide horretan askatutako base esfingoide batzuk lisosometatik ateratzen dira, eta berriz erabil daitezke CerS-en bidez zeramida berriak sintetizatzeko. Era berean, askatutako esfingosina, esfingosina kinasek fosforilatu dezakete esfingosina-1-fosfatoa sortzeko. Azken hori liasen bidez hidrolizatu daiteke PE eta gantz-aldehido bat askatuz. Bide horrek S1P-aren sintesiaren %50-90-aren erantzule dela uste da[82,85,86].



Irudia 16. Esfingolipido-metabolismoaren bideen irudikapen eskematikoa[86].

- Esfingomielina (SM) SPL ugariena da, eta zelula-mintzetan agertzen da, kanpoko aldean bereziki. Zeramida baten base esfingoidearen 1. posizioari fosfokolina bat gehituz eratzen da. Normalean, basea esfingosina bat izaten da, eta alboko kateak FA luzeez osatzen dira, kate ase edo monoinsaturatuekin[87].

  SM-en sintesia Golgi aparatuan eta mintz plasmatikoan gertatzen da **(16. Irudia).** Horretarako, zeramida bati, PC batetik datorren fosfokolina bat lotzen zaio, DAG molekula bat eta SM bat askatuz. Erreakzio hori esfingomielina sintasak (SMS) katalizatzen du. Bestalde, liso-esfingomielina baten azilazioaren bidez ere sintetizatu daiteke. Biosintesia Golgi aparatuan gertatzen denean, erretikulu endoplasmikoan sintetizatutako zeramida CERT-en bitartez Golgira garraiatzen da ATP kontsumitzen duen prozesu batean. Ondoren, ekoitzitako SM-en kantitate handi bat mintz plasmatikora garraiatzen da, besikulak garraiatzeko mekanismo baten bidez. Bestalde, SMasa-ek hidrolizatzen dituzte fosfokolina eta zeramida bat askatuz[81].

  SM-ak ugariagoak dira Golgi mintzean eta mintz plasmatikoetan, mitokondrioetan kontzentrazio baxuagoa dutelarik. Oro har, kolesteroletik gertu egoten dira, mintzetako lipido baltsak eratuz. Izan ere, hainbat ebidentziek bi molekula horien metabolismoak konektatuta daudela iradokitzen dute eta aldi berean, SM mailak zelulen barruko kolesterol banaketa kontrola dezakeela. Gainera, SM-ek kanal ionikoen formazioa eta funtzioa kontrolatzen dute. Bitxia bada ere, A2 fosfolipasaren jarduera inhibitzen dute, entzima garrantzitsua dena fosfolipidoen birmoldaketan eta eikosanoideen ekoizpenean. SM esfingolipido ugariena da nukleoan, eta bertan hainbat prozesutan

parte hartzen du. Izan ere, kromatinaren mihiztadura eta dinamikarako beharrezkoa da, eta bertako matrizearen osagai garrantzitsua ere da[82].

- Glikoesfingolipidoak bi taldetan banatzen dira: zerebrosidoak eta glangliosidoak. Zerebrosidoak, bereziki, monogluzikosilzeramidak dira, zeramida bati karbohidrato molekula bat gehituz eratuak. Egituran azukre ezberdinak izan ditzaketen arren, glukosa (glukosilzeramida – GlcCer) eta galaktosa (galaktosilzeramida – GalCer) dira ohikoenak. Egitura aldetik zeregin garrantzitsua dute mintzetan, burmuinean bereziki. SM-ak bezala, oro har, mintz plasmatikoaren kanpoaldean pilatzen dira, kolesterolarekin batera lipido baltsak eratuz. Glukosfingolipido konplexuen bitartekariak ere izan daitezke. Glukosilzeramida, nahiz eta ehun ezberdinetan dagoen, hala nola barean, eritrozitoetan eta nerbio-ehunean, larruazalaren osagai garrantzitsua da, eta axoien hazkuntzarako beharrezkoa da, bereziki garunean. Gainera, zelulen barneko mintz-garraiorako, zelulen hazkuntzarako eta biziraupenerako funtsezkoak direla frogatu da. Bestalde, galaktosilzeramidak nagusiki nerbio-ehunean daude, eta funtsezko eginkizuna betetzen dute oligodendrozitoen bereizketan eta mielinaren egituraren eraketan, egonkortasunean eta funtzioan. Bestalde, immunitate-sistemaren aktibatzaile indartsuak direla ikusi da[88,89]. Zelula-mailan, zerebrosidoak garrantzitsuak dira melanoma zelulen atxikipenean; izan ere, zelulen hazkuntzan eta bereizketan parte hartzen dute[89].

Esterol lipidoak konposatu poliziklikoez osatutako familia bat da, osagai ugariena kolesterola delarik. Kolesterola lau eraztunez osatutako molekula zurrun lau bat da, zeinak karbonodun albo-kate bat duen. Ehun guztietan agertzen da, eta bereziki mintz plasmatikoan pilatzen da (geruza bikoitzeko lipidoen %30-50). Modu librean, modu anfipatikoan edo kate luzeko FA batera esterifikatuta kolesterol-esterrak osatuz ager daiteke. Kolesterolak zeluletan funtsezko papera betetzen du, zelulen mintzen egitura-osagai garrantzitsua baita, bigeruzen jariakortasunean eragina duenak, fosfolipidoen artean tartekatzen baita. Gainera, hormona esteroideoen eta beste metabolito garrantzitsu batzuen aitzindaria da, D bitamina eta behazun-azidoak kasu. Izan ere, funtsezkoa da zelula-seinaleztapenerako, garraiorako, morfogenesirako, lipidoen xurgapen eta digestiorako, eta nerbio-eroapenerako, besteak beste[91]. Kolesterol esterrak ostera, molekula guztiz hidrofoboak dira eta mintzetan pilatu beharrean, lipido-tantetan pilatzen dira kolesterol biltegi modura.

Mebalonatoaren bideak esterol isoprenoideen biosintesia bultzatzen du, hala nola, kolesterola, hormona esteroideoak eta behazun-azidoak, eta bestetik isoprenoide ez-esteroideoena. Oso prozesu konplexua da hori, gutxienez 30 erreakzio entzimatikok hartzen baitute parte. Lehenik, mebalonato bitartekariaren sintesia lortzen da, ondoz ondoko bi erreakzio entzimatikotan gertatzen dena, azetil-CoA eta azetoazetil-CoA batuz HMG-CoA sintasaren eraginez. Ondoren, sortu berri den HMG-CoA erreduzitzen da HMG-CoA erreduktasaren bidez (HMGR)[92]. Azken urrats hori mugagarria da sintesi bide honetan. Zeluletan esterol-isoprenoide gutxi badago, esterolak erregulatzen dituen elementuarekin lotzeko proteinen (SREBP) transkripzio-faktoreak aktibatzen dira eta HMGR genearen transkripzioa sustatzen da. HMGR entzimaz gain, SREBP-ek mebalonato bideko entzima batzuen transkripzioa aktibatzen dute[93]. Bestalde, SREBP-ak fosfolipidoen sintesi-entzimen adierazpenaren gorakadarekin ere lotu dira[94].

Segidan, azido mebalonikoak bi fosforilazio jasaten ditu, bi isopreno sortuz. Horiek elkarrekin kondentsatzen dira eskualenoa emateko. Gero, eskualenoak hainbat erreakzio entzimatiko izaten ditu, azkenean kolesterola sortzeko.

Tesi honetan aztertzen ez diren beste lipido mota batzuk, prenol-lipidoak, sakarolipidoak eta poliketidoak dira.

Prenol-lipidoak, mebalonatoaren bidean sortzen diren isopentenil difosfatoren eta dimetilalil difosfatoren bost karbonodun aitzindarien kondentsatzioagatik sintetizatzen dira. Isoprenoide buztan bat dute, oinarri kinonoide bati lotuta. Karotenoideak lipido mota honen osagai garrantzitsuak dira, A bitaminaren eta antioxidatzaileen aitzindari bezala jarduten duten isoprenoide sinpleak baitira. Gainera, E bitamina eta K bitamina familia honen kide garrantzitsuak dira[83].

Sakarolipidoak, FA baten eta azukre molekula baten arteko lotura batez osatuta daude. Batez ere bakterioetan eta landareetan aurkitzen dira[83].

Poliketidoak azetilo eta propionilo azpiunitateen polimerizazioaren ondorioz sintetizatzen dira. Lipido mota honetako kide asko bigarren mailako metabolitoak eta animalia-produktuak dira. Horien artean, hainbat agente antimikrobiano, antiparasitarioak eta antikantzerigenoak daude[59].

2.1.2. Lipidoen izendapena

Lan honetan, notazio laburtua erabili da. Metodo horrek nomenklatura estandar eta praktiko bat ematen du, LIPID Maps terminologian oinarritzen dena. Lipido mota bakoitzak bere oinarria, buru polarra eta azukre molekula gehigarrien presentzia definitzen dituen izendapen bat izango du. Gainera, egiturari buruzko informazioa azilo-kateen deskribapenak ematen du, kate horien karbono eta insaturazio kopurua adierazten baita[83,95]. Beraz, lipido espezie bakoitza, bere lipido motaren laburduraren bidez izendatzen da, jarraian, kako artean karbono kopurua eta insaturazio kopurua, bi puntuz banatuak. Nomenklatura horretan, ordea, insaturazio mota (lotura bikoitzak edo hirukoitzak), posizioa eta geometria ez dira zehazten. Adibidez, hiru saturazio dituen 20 karbonodun gantz-azido luze bat FA(20:3) izendatuko litzateke.

Gainera, lipidoa bi FA kate dituen glizerofosfolipido bat baldin bada, lehenik 1. karbonoan (sn-1) esterifikatuta dagoen FA-a izendatzen da eta ondoren 2 karbonoan (sn-2) esterifikatuta dagoen FA-a, barra zeihar batez banandua. Beraz, 1. karbonoan FA(16:0), 2. karbonoan FA(20:4) eta hirugarren karbonoan fosfokolina buru polarra duen glizerol oinarri bat, PC(16:0/20:4) deituko litzateke. Batzuetan, ordea, ezinezkoa da bi FA kateak definitzea, eta bi lipido kateen karbono eta insaturazio guztien batura adierazten da. Kasu honetan, PC(36:4) bezala idatziko litzateke.

Glizerofosfolipidoak FA bat galdu badu, liso hitza erabiltzen da. Hau da, adibidez, LPE (18:0), lisofosfatidiletanolamina 18:0 FArekin esterifikatuta adierazten du. Teknologia hau erabiliz ezin da jakin glizerol eskeletoko zein karbonotan esterifikatzen den FA hori.

FA-k esterifikatua dituzten lipidoez gain, badira eter lipidoak, gantz-alhohol bat O-alkil lotura baten bitartez lotuta dituztenak, O batekin adierazten dena. Edo, O-alk-1-enilo lotura badute P batekin adierazten da[95]. Adibidez, etanolamina buru polarra duen lipido molekula bat, FA(18:0)-tik deribatzen den gantz-alkohol bat alkenilo lotura batekin erantsita duena, eta FA(20:4) bat esterifikatua sn-2 kokapenean, PE(P-18:0/20:4) izendatuko litzateke.

Esfingolipidoak, bere aldetik, sn-2 kokapenean d- edo t- izan daitezke, esfingoide basean duten hidroxilo taldeen arabera: d- di eta t- tri. Gero, base esfingoidearen karbono

kopurua:insaturazioak kako artean adierazten dira; ondoren, barra bat eta esfingoide basera atxikituta dagoen FA-ren (karbono:insaturazio) kopurua idazten dira[95]. Beraz, esfingomielina molekula bat, 18:1 dihidroxilo oinarri esfingoide batekin eta FA(18:2) sn-2 kokapenean, SM(d18: 1/18:2) izena izango luke.

Taula 3. Lan honetan identifikatu diren lipido moten laburdurak.

Lipido klasea	Laburdura
Gantz-azido askea	FFA
Glizerolipidoa	GL
Diglizeridoa	DG
Trigliceridoa	TG
Glizerofosfolipidoa	GPL
Azido fosfatidikoa	PA
Fosfatidilkolina	PC
Lisofosfatidilkolina	LPC
Fosfatidilkolina eterra	PC(P/O)
Fosfatidilserina	PS
Fosfatidiletanolamina	PE
Lisofosfatidiletanolamina	LPE
Fosfatidiletanolamina eterra	PE(P/O)
Fosfatidilinositola	PI
Fosfatidilglizerola	PG
Kardiolipina	CL
Esfingolipidoa	SPL
Zeramida	Cer
Esfingomielina	SM
Hexosilzeramida	HexCer
Esterola	
Kolesterol esterra	CE

2.2. Analisi lipidomikoak

Melanoma tumoreen izaera heterogeneoak zaildu egiten du guztiz zehatzak diren biomarkatzaileak eta tratamendu-aukerak aurkitzea. Gainera, prozesu kartzinogenikoaren aldakortasuna hain da handia, ezaugarri histologiko berdinak dituzten tumoreek genetika, proteomika, metabololomika eta epigenetika desberdinak dituztela, hazkuntza- eta metastasi-profil ezberdina aurkeztuz. Beraz, neoplasietan ematen diren aldaketa molekularrak hobeto ulertzeak berebiziko garrantzia du. Horren ildotik, zelulen lipido edukia eta horien metabolismoa aztertzeak interes handia piztu du azken hamarkadetan.

Lipidomika, lehen aldiz, 2003an definitu zen, lagin baten lipido edukia bereiztea eta kuantifikatzea helburu duen zientziaren arlo bat bezala. Gainera, molekula horiek beste biomolekula batzuekin (beste lipidoekin, proteinekin edo material genetikoarekin) duten elkarreragina identifikatzen du. Ordutik, analisi lipidomikoek etorkizun handiko eremu gisa eboluzionatu dute minbiziaren ikerketan eta biomarkatzaileen aurkikuntzan. Gaur egun, estrategia horiek maiz erabiltzen dira minbizi zelulak eta ehunak detektatu eta sailka ahal izateko, ehun normala eta tumorala ezberdintzeko asmoz[96–99].

Lipidoen aniztasun fisiko eta kimikoa dela eta, ez dago zelula edo lagin baten lipidoma osoa saiakera bakar batean aztertzen duen prozesu analitikorik. Azkenaldian, ordea, lipido kopuru zabala identifikatzea ahalbidetzen duten hainbat prozedura analitiko garatu dira. Eremu horren bilakaera azkarrak lotura estua izan du masa espektrometria tekniketan izan diren aurrerapen handiekin. Azterketa hauek NMR (erresonantzia magnetiko nuklearra), Raman espektroskopia eta teknika espektroskopiko batzuk erabiliz egin daitezkeen arren, nagusiki masa espektrometria (MS) metodoetan oinarritzen dira. Masa espektrometria zuzenean laginetan burutu daiteke edo kromatografia likidoarekin konbinatu. Analisi lipidomikoen lan-fluxu arrunt batek hainbat pausu biltzen ditu, hala nola lagin biologikotik lipidoak erauztea, balizko bereizketa kromatografikoa, ionizazio leuna eta MS-n oinarritutako analisia, eta datuen prozesamendua.

Lan honetan, bi metodologia lipidomiko ezberdin erabili ditugu. Horretarako azterketa lipidomikoetan gehien erabiltzen diren masa espektrometria estrategiak erabili ditugu: ESI-MS/MS tandem metodologia UHPLC-ri moldatuta, eta beste alde batetik MALDI-MS metodologia.

2.2.1. UHPLC-ESI-MS/MS metodologia

Estrategia honetan, ikasitako zelula lerroen lipido erauzkinak behar ziren, beraz, lehenengo urratsa lipido erauzketa izan zen. Erauzketa-metodo egokiena aukeratu aurretik, laginaren erauzketa-ehunekoa ebaluatu behar da, eta, horretarako, berreskuratze-esperimentu bat egin behar da, hainbat metodologia konparatuz. Horretarako, erauzi aurretik laginei, lipido-estandarren kopuru ezagun bat gehitzen zaizkie. Estandar horiek azterketa-laginetan aurkituko diren lipido-familiak adierazten dituzte. Bestalde, kontrol baldintza bat behar da. Horri, lipido erauzketa eta gero gehitzen zaio lipido-estandarrak. Horrela, kontrol lagin horretan lortzen den estandarren intentsitatea, gehitu diren estandar guztien kopuruaren adierazgarri izango litzateke, ez baita ezer galdu erauzketa egiterakoan. Gero, laginetako estandarretan lortutako intentsitatea kontrol-baldintzaren estandarren intentsitatearekin alderatzen da. Horrela, intentsitate-diferentziak erauzketatik errekuperatzen den laginaren ehunekoa adierazten du, hau da, errekuperazio-tasa. Bligh & Dyer metodoak berreskuratze-ehuneko hobeak erakusten ditu, oro har, isopropanolarekin egindako proteinen hauspeatze-metodoa baino **(4. taula)**. Beraz, Bligh & Dyer-ren metodoa lagin biologiko txikietatik lipidoak erauzteko metodo estandar bezala aukeratua izan zen.

Taula 4. Lipido-estadarren berreskuratze tasak (%) lagin baten lipidoak erauzteko Bligh & Dyer metodoa eta isopropanol bidezko hauspeatzea erabiliz.

Lipido mota	Lipido-estandarra	Berreskuratze tasa (%)	
		Bligh & Dyer	Isopropanol
Glizerolipidoak	TG(14:0-16:1-14:0)	97	84
	TG(16:0-18:0-16:0)	94	66
	TG(15:0-18:1-15:0)	93	88
	TG(17:0-17:1-17:0)	100	88
Glizerofosfolipidoak	PC(17:0/14:1)	102	95
	LPC(17:1)	82	91
	PI(17:0/14:1)	46	86
	PG(17:0/14:1)	70	95
Esfingolipidoak	SM(d18:1/12:0)	96	87
	Cer(d18:1/25:0)	105	116
	Cer(d18:1/12:0)	91	91
	GlcCer(d18:1/12:0)	94	93
	LacCer(d18:1/12:0)	89	103

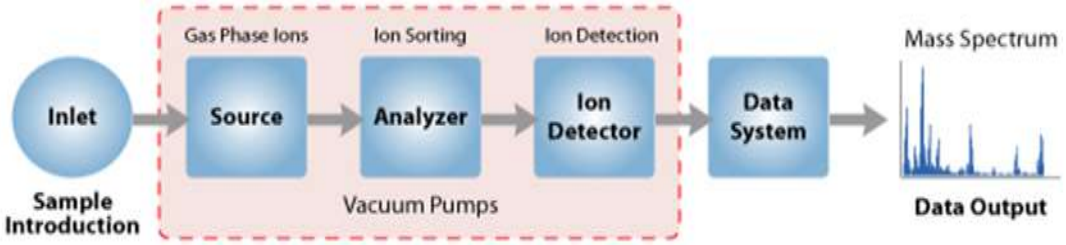
Bligh & Dyer metodoan, bi faseko banaketa lortzen da, kloroformo eta metanol-ur disolbatzaileak erabiliz. Disolbatzaile ez-polarrean, hau da, kloroformoan, lipido hidrofobikoak diluitu egiten dira, eta, beraz, erauzi; aldiz, lipido polarrak metanol disolbatzaile polarrean kontzentratzen dira, zeinak lipidoak mintzeko proteinetara lotzen dituzten hidrogeno-loturak edo indar elektrostatikoak hausten dituen. Gainera, alkohol-disolbatzaileak fosfatidasa eta lipasa askoren inaktibazioa eragiten du, lagineko lipidoen degradazio entzimatikoa saihestuz[100]. Beraz, prozedura horri esker, kloroformo fasean glizerola, esteroideak, zeramidak eta esfingomielinak dituzten lipidoak erauzten dira; lipido polarrak, berriz, hala nola, PI fosforilatuak, glukolipido konplexuak, bereziki gangliosidoak, eta osagai ez-lipidikoak (azukreak adib.) fase urtsuan geratzen dira, eta deuseztatzen dira[101].

Aurreko atalean azaldu den bezala, lipidoma zelularra lipido nahasketa oso konplexua da, lipido mota bakoitzaren barnean lipido espezie ugari daudela kontutan hartuz. Beraz, MS-aren aurretik sarritan lipidoen familien araberako bereizketa kromatografikoa burutzen da, molekula isobarikoak behar bezala identifikatzea zaila izan baitaiteke. Beraz, ikerketa lipidomikoetan, sarritan MS-ari akoplatutako analisi kromatografiko ezberdinak erabiltzen dira[96–99].

Metodologia honen hurrengo urratsa lipido mota ezberdinen bereizketa kromatografikoa da. Zehazki, ultrapresio altuko kromatografia likidoa (UHPLC) oso teknika onuragarria da lipido anfipatikoak euren propietate fisiko-kimikoen arabera bereizteko. Izan ere, alderantzizko faseko UHPLC-a erabiltzen denean, klase bereko lipidoak lipofiliaren arabera bereiz daitezke, zeina molekularen azilo-kateen luzeraren eta lotura bikoitzen kopuruaren arabera zehaztuta dagoen. Adibidez, elusio denbora laburragoa da azilo kate laburrak eta azido poliinsaturatuak dituzten lipidoentzat[102]. Gainera, UHPLC estrategia oso komenigarria da fosfolipidoak azpi-klaseetan bereizteko(perrotti). Kromatografoa espektrometrora akoplatzen denez, nahastearen osagaiak euren atxikipen-denboraren arabera banandu, espektrometroan sartu eta MS-n oinarritutako analisien bidez identifikatzen dira.

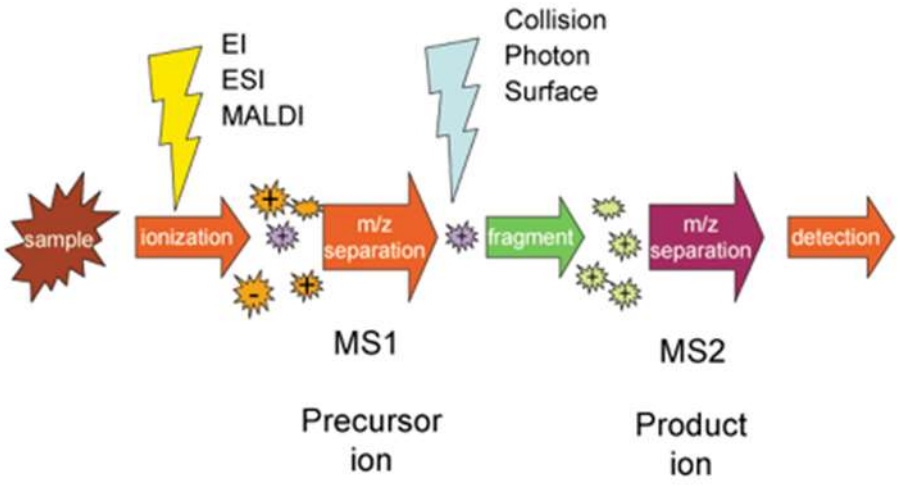
Masa espektrometria teknikek kargadun molekula (ioi) desberdinak identifikatzen dituzte, haien masa-karga erlazioaren arabera (m/z), analitoen informazio estrukturala zein kuantitatiboa emanez. Espektrometro baten funtsezko atalak ioi-iturria, masa-analizagailua, detektagailua eta

datu-sistema dira[103]. Erauzitako lipidoak, UHPLC teknikaren bidez aldez aurretik bereizita edo ez, ioi-iturrian sartzen dira eta bertan ionizatzen dira, karga positiboa edo negatiboa hartuz. Gero, ioiak laginketa-konotik igarotzen dira, eta analizagailura iristen dira. Analizatzailearen helburua sortutako ioiak beren karga-masa-erlazioaren arabera bereiztea da. Adibidez, laupolo-hegaldi-denbora-analizagailua (Q-TOF) erabiltzen denean, laupoloak ioiak aukeratzen ditu eta talka-gelaxkara bidaltzen dira argoiaren bidez zatitzeko. Molekula horiek hegaldi-denboraren araberako analizagailura iristen dira, eremu elektriko bat aplikatzen duenak ioi guztiak potentzial berera bizkortzeko, eta ioi bakoitzak detektagailura iristeko behar duen denbora neurtzen da, bere masaren araberakoa dena. Masa altuagoa duten ioiak beranduago iristen dira detektagailura. Azkenik, detektagailuak ioien masari eta nahastean duten ugaritasunari buruzko informazioa erregistratzen du, gero datu-sistemara bidaltzen dena, non grafikoki masa-espektro gisa irudikatzen den. Grafikoaren tontor bakoitzak nahasketan agertzen den masa espezifiko bat irudikatzen du, eta tontor bakoitzaren zabaltasunak ioi horren ugaritasun erlatiboa adierazten du.



Irudia 17. Masa-espektrometro baten osagaien irudikapen eskematikoa[103].

Lipidoen ikerketaren arloan egindako aurrerapen handiak, hein handi batean, ionizazio leuneko MS tekniketan lortutako aurrerapenei zor zaie. Horien barne ESI (elektroesprai bidezko ionizazioa) eta MALDI (laser bidezko desortzioa eta ionizazioa matrize bidez lagunduta) daude. ESI-k energia elektrikoa erabiltzen du erauzketa-disolbatzailean edo UHPLC-ko fase mugikorrean dauden ioiak aerosol baten moduko gas-fase batera transferitzeko. Disolbatzailea lurruntzeko eta aerosola lortzeko, soluzioa, potentzial handiko kuartzozko kapilar baten bidez lainoztatzen da; karga duten tanten disolbatzailea lurruntzeko, nitrogeno beroko zurrusta bat erabiltzen da. Azkenik, disolbatzaile gabeko lagin-ioiak laginketa-konotik igarotzen dira, eta analizagailura eta detektagailura iristen dira. UHPLC-ESI-MS/MS metodologiak neurri handi batean kantitate txikian agertzen diren lipidoak detektatzeko sentsibilitatea eta zehaztasuna areagotzen du. MS/MS tandem teknika erabiliz gero, molekularen egiturari buruzko informazio gehigarria eman daiteke[104]. Hemen, bi analizagailu ezberdin erabiltzen dira; lehenik, ioi aitzindaria detektatu eta zatitzen da; ondoren, zatitutako ioiak bigarren masa analizagailura sartu eta detektatzen dira. Horrela, bi analizagailuetan lortutako emaitzek, lipido aitzindariaren egitura era eraginkorrago batean identifikatzen laguntzen dute[96,98]. Garrantzitsua da azpimarratzea ESI dela soluzio batean dauden lipidoak aztertzeko lehentasunezko ionizazio-metodoa[105].



Irudia 18. Tandem MS/MS estrategiaren lan-fluxua eskematikoki adierazita[104].

2.2.2. MALDI-MS metodologia

Lan honetan erabili zen bigarren metodologian zelula-mintz funtzionalez osatutako mikroarraietan MALDI-MS erabili zen. Horretarako, hasteko, zelula lerro ezberdinen mintz zelularrak erauzi eta immobilizatu ziren mikroarrai bat osatuz. Azkenik, azterketa lipidomikoa burutzeko, MALDI-MS metodologia aplikatu zen.

MALDI-MS teknika maiz erabiltzen da biomarkatzaileak aurkitzeko. MALDI-MS laserrean oinarritutako ionizazio leuneko teknika bat da, non pisu molekular txikiko matrize organiko baten geruza mehe bat aplikatzen den laginaren goialdean. Matrize horrek laser erradiazioa xurgatzen du eta ioi molekularrak eta ioi zatikatuak igortzen ditu. Prozesu analitiko horretan urrats kritikoetako bat lagina prestatzea da. Izan ere, matrizeak hautatu eta jartzeko era funtsezkoa da. Frogatu da matrizeak jartzeko metodorik onena matrizea sublimatzea dela, laginean zehar banaketa erregularra bermatzeko, eta aldi berean, kristal txikiak osatzen dituzten matrizeak erabiltzea. MBT (2-merkaptobenzotiazola) eta DAN (2,5-diaminonaftalenoa), hurrenez hurren, ioi positiboak eta negatiboak detektatzeko, espezifikotasun handia, eta lipido espezie desberdinen identifikazio zehatza erakutsi dute[106]. Metodologia analitiko hori egokia da lipido polarrak, fosfolipidoak eta esfingolipidoak barne, eta lipido ez-polar batzuk, hala nola, DG-ak eta TG-ak, detektatzeko[98,107].

2.3. Minbizi zelulen metabolismoa

Minbizi zelulek aldaketa molekularrak jasaten dituzte, minbizi-fenotipoaren eskurapenean eta mantenuan laguntzen dutenak. Horrek abantaila ematen die ingurugiro tumoralak sortutako baldintza estresagarrietan hazteko eta bizirauteko[108]. Tumore-zelulen behar anabolikoak eta energetikoak babesteko, zelulen bidezidor metabolikoak zehaztasunez birkonektatzen da, aldaketa translazional zein post-translazionalen bitartez erregulatuta dagoena.

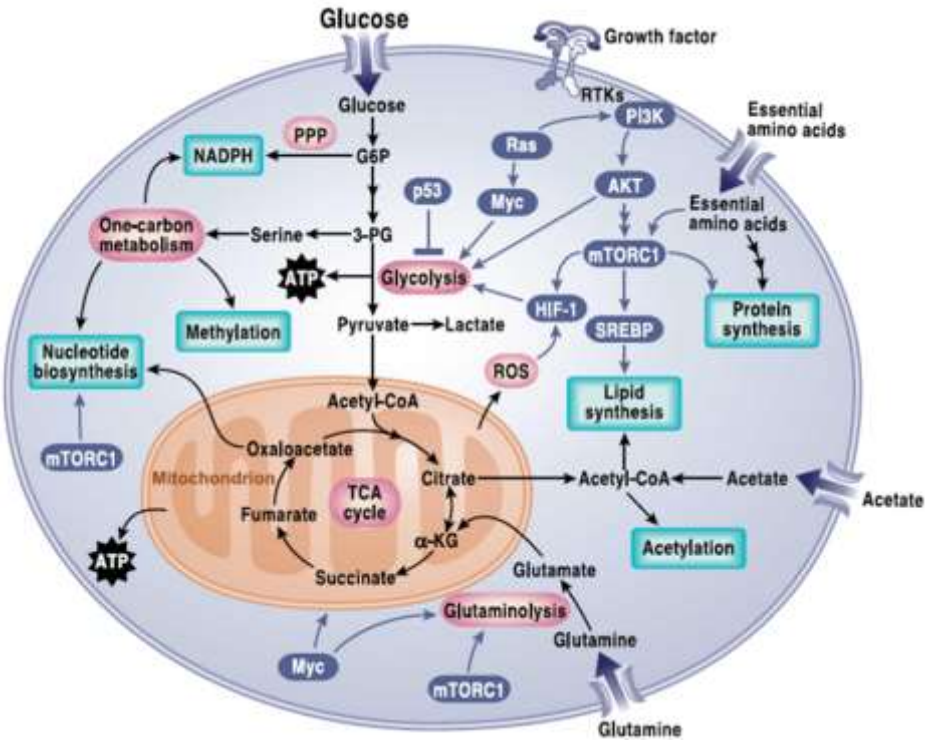
Laburbilduz, baldintza fisiologikoetan, zelula osasuntsuetan xurgatutako glukosa nagusiki pirubato bihurtzen da zitosolean, eta fosforilazio oxidatiboaren (OXPHOS) bidez metabolizatzen da mitokondrietan, 36-38 ATP molekula sortuz glukosa molekula bakoitzeko[109].

Aitzitik, zelula gaiztoek ehun arrunten erantzun hipoxikoa erabiltzen dute, oxigenoaren eskuragarritasuna edozein dela ere **(19. Irudia)**. Hori, Warburg efektua bezala definitzen da, minbizi zeluletan deskribatutako lehen alterazio metabolikoa da, eta XX. mendearen lehen erdian Otto Warburgek deskribatu zuen. Modu esanguratsuan, Warburg efektuak minbizi zeluletan glukosaren metabolismoaren bide nagusia fosforilazio oxidatibotik hartzidura laktikora mugitzen dela baieztatzen du, ATP molekulen ekoizpena nabarmen murriztuz[110–112]. Melanoma konkretuki, minbizi oso glukolitiko gisa definitu da; izan ere, melanoma zelulek xurgatutako glukosaren %60-80 laktato bihurtzen dute normoxian, eta %90-ra edo gehiagora igotzen da hipoxia egoeretan[113]. Tumore zeluletan ikusitako plastikotasun metabolikoaren ondorio fisiologikoak alterazio bioenergetikoak eta biomolekulen sintesi areagotua dira. Horrela, glukosatik eratorritako karbonoak ez dira energia lortzeko erabiltzen, makromolekula berriak sortzeko baizik, eraikuntza-bloke biosintetiko gisa, hala nola, lipidoak, aminoazidoak eta nukleotidoak sintetizatzeko.

Duela gutxi arte, minbizi zelulek mitokondioak kaltetua zituztela uste zen, eta, beraz, elektroien garraio kateak ezin zuela ATPrik sortu, zelula horiek glukolisi aerobikoa erabiltzearen arrazoia hori zela sinetsita. Aitzitik, mitokondrioek behar bezala funtzionatzen dutela eta glukosaren ordez glutamina dela azido trikarboxilikoen zikloaren (TCA) karbono hornitzaile nagusia frogatu da[110–112]. Glutamina energia iturri garrantzitsutzat jotzen da hazten ari diren zeluletan. Gainera, glutaminak lipidoen sintesirako beharrezkoak diren karbonoak eta aminoazidoen, nukleotidoen eta lipidoen sintesirako beharrezkoak diren amino-nitrogenoak ere hornitzen ditu[110,114,115].

Minbizi zelulen beste energia iturri bat gantz azidoen oxidazioa da. Molekula horiek erreakzio kataboliko batzuen bidez laburtzen dira, TCA zikloa berrezartzeko erabiltzen den azetil-CoA sortuz, eta karbohidratoek sortzen duten ATP bikoitza sortuz, eta NADPH-a ekoiztuz. Energia-iturri horren erabilera elikagai eta oxigeno eskuragarritasuna urria denean nabarmentzen da. Izan ere, gantz azidoen oxidazioaren erabilera maila altuenak tumore erasokorrekin erlazionatu dira[115,116].

ATP-az gain, NADPH-a ere beharrezkoa da minbizi zeluletan areagotuta dauden bide anabolikoak mantentzeko. Gainera, NADPH-ak minbizi zelulak estres oxidatibotik babesten ditu. Gantz azidoen oxidazioaz (FAO) gain, pentosa fosfatoen (PPP) bideak glukosaren oxidazioaren ondoren NADPH-a ere ekoizten du[117].



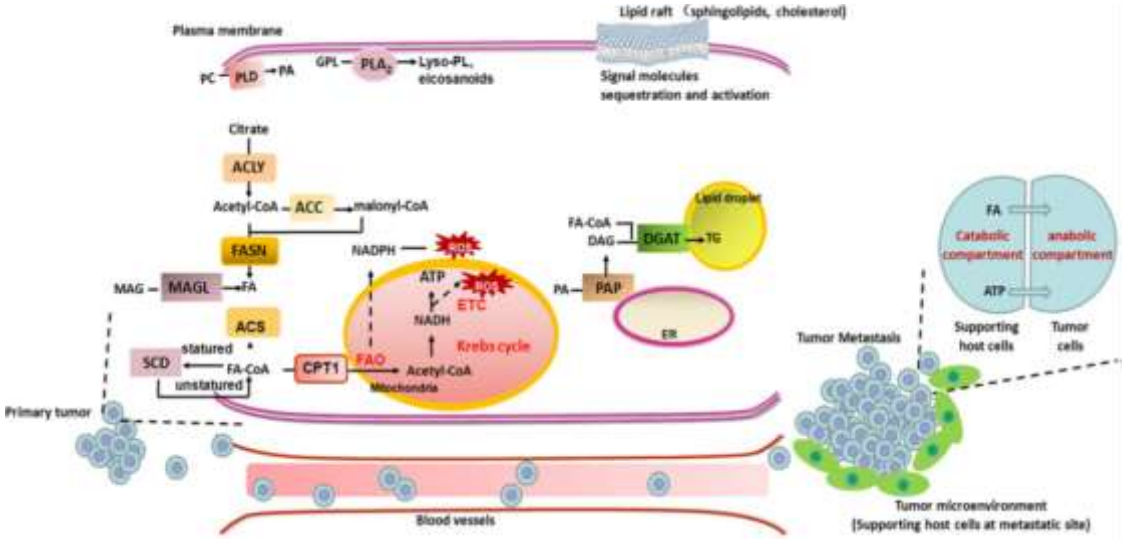
Irudia 19. Minbizi-zeluletako bidezidor metabolikoa erregulatzen duten seinaleztapen bideen irudikapen eskematikoa[108]. Zelula tumoraletan bide metaboliko desberdinen aktibazio aberranteak lipido, nukleotido eta proteinen biosintesia areagotzen du. Glukosaren xurgapena eta fluxu glukolitikoa handitzeaz gain. Laburdurak: PPP: pentosa fosfatoen bidea; G6P – Glukosa-6-fosfatoa; 3-PG -3-fosfoglizeratoa; ATP-adenosina trifosfatoa; a-KG – α-zetoglutaratoa; RTK-tirosina kinasa hartzailea.

2.3.1. Lipidoen metabolismoa eta minbizia

Lipidoen metabolismoan ematen diren aldaketek garrantzia irabazi du azken urteotan. Frogatu da bai *de novo* biosintesiak bai makromolekula horien oxidazioak gora egiten dutela minbizian. Lipido metabolismoan ematen diren aldaketen ondorioz, energia sorrera eten egiten da, zelulak seinaleztapen bideak aldatzen dira, zelula-mintzetan egitura-alterazioak daude eta geneen adierazpen eta proteinen banaketa aberrantea ematen da. Aldaketa horiek guztiek funtzio zelular esanguratsuetan eragina dute, hala nola, hazkuntza, apoptosia, autofagia, droga eta kimioterapiekiko erresistentzia, besteak beste[49].

Zelula gaiztoek lipidoen premia handia erakusten dute, kolesterola barne, eta, beraz, bai sintesiak eta bai molekula horien xurgapenak minbizian gora egiten dute. Gainera, minbizi ezaugarritzat jo da zelula horien barruan lipido tantak biltzea. Melanomak, bereziki, FA-en premia handia erakusten du bere progresio eta metastasirako. FA-k FAO-an energia ekoizteko edo mintzetan biltzen diren fosfolipidoen sintesirako erabiltzen dira, beste funtzio batzuen artean. Zelula erasokorrek lipidoen biosintesian, katabolismoan eta zelula barneko lipidoen biltegiratzean inplikatutako geneen adierazpen-maila altuagoak erakusten dituzte. Gainera, lipidoen metabolismoak prozesu metastatikoaren lehen urratsetan ez ezik, urrunerako barreiaduran parte hartzen duela frogatu da. Izan ere, prozesu horren blokeo farmakologiko edo molekularra siRNA-k erabiliz, apoptosi zelularra eta tumore primarioen bolumena murriztea ekartzen du[114,118].

Minbizi zelulen aldaketa metabolikoek SREBP transkripzio-faktorearen jarduera aberrantea bultzatzen dute, gantz azidoen, fosfolipidoen, triglizeridoen eta kolesterolaren metabolismoaren erregulatzaile nagusia. Izan ere, SREBP-ek lipidoen metabolismoa kontrolatzen du transkribapen, itzulpen eta itzulpen ondoko mailetan. Transkripzio-faktore horrek hainbat entzima lipolitiko eta lipogenoren adierazpenaren eta jardueraren erregulazio positiboa sustatzen du. Adibidez, FAS (gantz-azido sintasa), ACC (azetil-CoA carboxilasa), ACLY (ATP zitrato liasa) eta SCD (estearoil-CoA desaturasa) entzimen erregulazio positiboa minbizi askotan behatutako ezaugarri arrunt bat da, melanoma barne. **20. irudian** adierazten den bezala, entzima horiek zuzenean FA-en *de novo* biosintesian eta FA-en molekula berrien eraldaketan parte hartzen dute, karbono katea luzatuz eta insaturazioak sartuz. Nabarmentzekoa da entzima horien inhibizio farmakologikoak minbizi zelulen inbasioa, migrazioa eta biziraupena murriztu egiten dituela *in vitro*[114,119].



Irudia 20. Minbizi zelulen lipido metabolismoan ematen diren aldaketen irudikapen eskematikoa[120].

Hasiera baten FA-en sintesia eta oxidazioa bateraezinak diren prozesuak izan arren, minbizi zeluletan bata besteaz baliatuz batera existitzen dira. FA-en oxidazioak zeluletan azetil-CoA metatzea eragiten du, eta horiek FA-en sintesia hasteko erabil daitezke. Gainera, oxidazioan sortutako ATP eta NADPH molekulak helburu anabolikoetarako erabiltzen dira. Zelulek lipido toxikoak hidrolizatzen dituzte horiek deuseztatzeko, eta, aldi berean, FA-en sintesiak zelulen eraldaketa gaiztorako beharrezkoak diren espezie lipidiko zehatzak sortzen ditu[120].

Fosfolipidoen azpimota ezberdinen metabolismoa ere aldatu egiten da minbizian, fosfolipidoen biosintesia estimulatzen baita, eta horrek zelula tumoraletan horien edukia handitzen du[121,122]. Ebidentzia askok Kennedy bidean parte hartzen duten entzimak tumore ehunetan gehiago adierazten direla iradokitzen dute. Zehazki, GPAT2, prozesu mailakatu horren lehen erreakzioa kontrolatzen duen GPAT entzimen familiako kide bat, gainadierazita agertzen da minbizi ehun batzuetan[77]. Kolina kinasa (CK), PC-en biosintesiaren lehen urratsaren arduraduna dena, presentzia eta aktibitate handiagoa du minbizi zeluletan[123]. Tumoreen garapenean, progresioan eta barreiadura metastatikoan parte hartzen du hainbat minbizi motatan, melanoman barne[122]. Horren ildotik, garapen klinikorako CK-ren inhibitzaile ugari aztertzen ari dira, *in vitro* eta *in vivo*

eraginkortasun handia erakutsi ondoren[55,124]. PC-en biosintesi tasa altua mantentzeko, kolina askearen xurgapena ere estimulatzen da. Zelulen barnean kolinaren garraioan parte hartzen duten proteinak, zelula minbizi ezberdinetan, melanoman barne, gainadierazita agertzen dira. Izan ere, proteina horiek hainbat mekanismoren bidez inhibitzeak tumore-zelulen hazkuntza, apoptosiaren areagotzea eta tumore-progresioaren blokeoa eragiten du[123]. Bestalde, PC-en katabolismoa ere handitzen da tumore zeluletan. Hori oso garrantzitsua da tumoreen progresioan, eta A, C eta D fosfolipasen bidez egiten da, bigarren mezulari oso garrantzitsuak sortuz, hala nola, DAG, PA, LPA, FFA, Lyso-PC, kolina askea eta fosfokolina[121].

Plasmalogenoek minbizian duten paper zehatzaren ebidentziarik ez dagoen arren, lan askok ehun minbiziduneta eter lipidoen biosintesian parte hartzen duten zenbait entzimen gehiegizko adierazpena erakusten dute. Gainera, ADAPS hainbat minbizi motatan gainadierazten dela frogatu da. Horren ondorioz, alkil eter lipidoen mailak igotzen dira, minbiziaren progresioa erraztuz[74]. Izan ere, entzima horren inaktibazioa eta blokeoa minbiziaren progresio motelago batekin lotu izan da. Aldiz, bere gainadierazpenak, eter lipidoen edukia handitzen duenak, tumore zelulen hazkuntza, mugikortasuna eta biziraupena handitzen duela frogatu da[70,125].

FAO mitokondrioetan ez ezik, peroxisometan ere gertatzen da. Mekanismo oxidatiboa bi organuluetan berdina den arren, organulu bakoitzean inplikatutako entzimak ezberdinak dira, eta FA ezberdinak metabolizatzen dituzte. Izan ere, gantz azido adarkatuak eta kate oso luzekoak (>26 karbono) peroxisometan bakarrik hidroliza daitezke. Bertan lehen erreakzio oxidatiboak gertatzen dira, kate luzeak txikiago batzuetan hautsiz, eta ondoren mitokondrioetara garraiatzen dira prozesuarekin jarraitzeko[74,75].

Beste alde batetik, Lands zikloan parte hartzen duten liso-fosfolipidoen transferasa espezifikoak, (LPAT) eta PLA_2, gainadierazita daude hainbat minbizi motatan, hala nola, gibeleko, koloneko, prostatako, biriketako, urdaileko eta bularreko minbizietan[77]. Seinaleztapen molekula garrantzitsuen ekoizpena handitzeak, hala nola, liso-PL eta gantz-azido askeak, hazkuntza, migrazio eta ahalmen metastatiko handiagoan bihurtzen da[96].

Esfingolipidoei dagokienez, hainbat minbizietan glukosilzeramida sintasaren (GCS), SM sintasaren (SMS), zeramida kinasaren (CERK), zeramidasa azidoaren (aCDase) eta/edo esfingosina kinasaren (SK) jarduera areagotuta izatea ohikoa da. Egoera horrek SPL-en adierazpen maila handitzen du. Oro har, zeluletan zeramidak metatzea apoptosia sortzen du, nahiz eta berriki zeramida motaren araberakoa dela frogatu den. Adibidez, zelula minbizidunek 18 karbonodun zeramida maila baxua dute, CerS1 adierazpenaren murrizketaren ondorioz. Aldiz, CerS2 adierazpenaren gorakadaren ondorioz kate luzeko (22-24 karbono) zeramidak minbizi-zeluletan metatzen dira[84,85]. Melanoman bereziki, CerS6-ren adierazpen baxua pronostiko txar batekin lotu zen. Entzima horrek melanomaren progresioan duen garrantzia frogatzeko, bere adierazpena hainbat zelula lerroetan blokeatu zen. Horren ondorioz, zelulek progresio eta inbasio handiagoa erakutsi zuten[126]. Gainera, 16 karbonodun zeramiden ekoizpena eta metaketa eragiten duten CerS5/6-en *in vitro* gainadierazpena zelula minbizidunen apoptosia areagotzen du. Bitxia bada ere, tratamendu kimioterapeutiko eraginkorrek, kate luzeko zeramiden metaketa sustatzen dute, eta CerS4, 5 eta 6-ren adierazpena igotzen da[85].

Bestalde, melanomak, beste minbizi batzuen artean, zeramidasa azidoetan (aCDasa) maila altuak erakusten ditu. Lan askotan aCDasa lisosomikoak melanoma zeluletan ugaritze fenotipotik inbaditzailerako trantsizioan parte hartzen duela erakutsi da. Horren ildotik, melanoma zeluletan, aCDasa-ren espresio handiago bat hazkuntzarekin erlazionatu da.

Adierazpen murriztua, aldiz, mugikortasun eta gaitasun inbaditzaile handiago batekin erlazionatu da[127]. Gainera, aCDasa-ren gehiegizko adierazpenak kimioterapeutikoei aurkako erresistentzia sortzen duela frogatu da, entzima horiek prozesu kantzerigenoan duten garrantzia nabarmenduz[128].

SM-k minbizian duten zeregina dela eta, SM sintasaren (SMS) erregulazio negatiboa pronostiko txarrarekin erlazionatu da melanoman. Egoera horrek melanoma primario eta metastatikoetan SM mailak murriztea eragiten du[129]. Bestalde, SMasa azidoak melanomaren prozesu metastatikoan funtsezkoak direla frogatu da[84].

Minbizian jarduera handiagoa erakusten duen beste bide lipogeniko bat, mebalonatoaren bidea da. Lehen azaldu den bezala, SREBP transkripzio faktorearen jarduera areagotuaren ondorioz, zenbait minbizi motak mebalonato bideko entzimen adierazpena eta jarduera handiagotuta dutela erakutsi da. Bide hori aktibatzen denean, zelulek kolesterola sintetizatzen dute, ehun ezberdinetan pilatzen dena[130]. Kolesterol-esterrak, bestalde, azil-CoA:kolesterol azil transferasaren (ACAT) bidez sintetizatzen dira. Hori, minbizi ehunetan gainadierazita aurkitzen da, eta gainera, minbiziaren progresioarekin korrelazionatzen da, giltzurruneko minbizian adibidez[131]. Estatinak, klinikan oso erabiliak dira eta plasmako kolesterol mailak murrizten dituzte. Estatinen eraginez, minbizi-zelulen hazkuntza murrizten da, apoptosia eragiten da eta minbizi zelulak eragile kimioterapeutikoekiko sentikorragoak dira *in vitro*. Izan ere, agente kimioterapeutikoekin batera estatinak emateak eraginkortasun handia erakutsi du. Hala ere, ondorio horiek oraindik aztertzen ari dira, badirudi erantzuna minbizi motaren araberakoa dela[132].

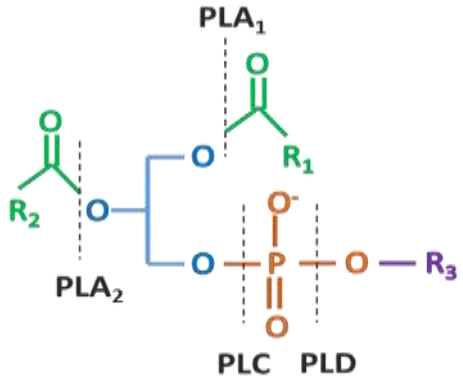
Laburbilduz, minbizi zelulen metabolismoa, eta zehazki lipidoen metabolismoa, aldatu egiten dela frogatu da. Aldakuntza metaboliko horiek minbizi zelulek dituzten ezaugarri gaiztoak bereganatzen laguntzen dute. Beraz, lipidoen metabolismoan parte hartzen duten funtsezko entzimen inhibizioa etorkizun handiko tratamendu aukera izan daiteke.

3. D Fosfolipasa eta minbizia

Fosfolipasa entzima-familiak glizerol molekula duten fosfolipidoen katabolismoan hartzen du parte. Familia osatzen duten entzimak lau multzo nagusitan sailkatzen dira, eta horietako bakoitzak fosfolipido molekula ezberdinak hidrolizatzen ditu posizio jakin batean **(21. Irudia)**. Entzima horiek ezinbestekoak dira baldintza fisiologikoetan zein patologikoetan, mintz plasmatikoaren birmoldaketarako eta homeostasirako beharrezkoak baitira. Izan ere, sarri minbiziarekin eta beste gaixotasun batzuekin lotu izan dira, entzima horien jardueraren ondorioz lipido bioaktiboak sortzen baitira, zelula barneko eta zelula arteko seinaleztapen bideak arautuz. Ondorioz zelulen hazkuntza, biziraupena, migrazioa, besikulen trafikoa eta heriotza arautzen dituzte besteak beste. Hain zuzen ere, familia horretako kide askok hainbat minbizi motatan euren adierazpena handituta dutela ikusi da[133].

Fosfolipasa ezberdinen entzima-jarduera:

- PLA: A_1 eta A_2 fosfolipasek fosfolipidoen ester lotura sn-1 edo -2 kokagunean moztu dezakete, hurrenez hurren, gantz azido aske bat eta liso-fosfolipido bat askatuz.
- PLB: PLA_1 eta PLA_2-ren jarduera berdina du.
- PLC: fosfatoa eta glizerola sn-3 kokagunean batzen dituen lotura hidrolizatzen du, DAG molekula bat eta fosforilatutako buru polarra askatuz.
- PLD: buru polarra eta fosfatoaren arteko lotura mozten du, buru polar aske bat eta PA molekula bat askatuz.



Irudia 21. Fosfolipasa ezberdinen hidrolizazio lekuak

Ugaztun zeluletan PLD azpiklasea, sei isoforma ezberdinez osatuta dago. Orain arte, PLD1 eta PLD2 aztertu dira gehien. Biek jarduera katalitiko bera erakusten duten arren, hau da, nagusiki PC bat hidrolizatzen dute PA eta kolina aske bat sortuz, beraien ehun eta kokapen azpizelularra ezberdinak dira. PLD1 nagusiki, mintz perinuklear ezberdinen barneko aldean kokatzen da. Zehazki jariaketa pikorretan, endosometan, lisosometan eta Golgi aparatuan. Aktibatu ondoren, mintz plasmatikora garraiatzen da. Bertan bere jarduera hidrolitikoa burutzen du mintzen garraioan, mitosian eta seinaleen transdukzioan eraginez. PLD1 isoforma minbiziarekin, gaixotasun tronbotikoekin eta autoimmunitatearekin erlazionatuizan da. Bestalde PLD2, mintz plasmatikoan kokatzen da nagusiki eta jarduera katalitiko indartsu batekin lotu izan da. Izan ere, PLD2-aren lipasa jarduera zitoeskeletoaren berrantolaketarekin, jariaketa arautuarekin eta ziklo zelularraren kontrolarekin erlazionatu da. PLD1-aren antzera, PLD2-ak ere minbiziaren sorreran

parte hartzen duela jakin da. Horrez gain, hipertentsioan eta Alzheimer gaixotasunarekin zerikusirik duela ere jakin da[133].

Orain arte deskribatutako isoformez gain, ugaztun zeluletan PLD3, PLD4, PLD5 eta PLD6 isoformak ere aurkitzen direla deskribatu da. Horien funtzioei buruz informazio asko egon ez arren PLD3, PLD4 eta PLD5 isoformek mintza zeharkatzen duen domeinu baten bidez mintz plasmatikoan finkatuta daudela frogatu da. PLD6 aldiz, mitokondria mintzaren kanpoko azalean aurki daitekeen proteina dimeroa bezala deskribatu da[133].

PLD1-en eta PLD2-ren sekuentziek %50eko homologia dute, nahiz eta PLD2 laburragoa izan. Amankomunean duten egituran, HKD fosfatidiltransferasaren domeinu kontserbatua bikoiztuta da ageri da. Sekuentzia honek bi entzimen toki katalitikoa kodifikatzen du. Domeinu honen bereizgarria HxKx4Dx6 aminoazidoen sekuentzia da, histidina, lisina eta azido aspartikoarekin, non x edozein aminoazido izan daitekeen. Gainera, PLD1 eta PLD2-ren amino eremu terminalean, PX (Phox Homologia) eta PH (Pleckstrina Homologia) domeinuen tandema dago. Tandem horrek mintza eta lipidoen arteko elkarrekintzan parte hartzen du eta entzimen kokapen azpizelularra kontrolatzen du. Hala ere, domeinu horiek ez dute entzimen jarduera katalitikoan eragiten[134]. Gainerako PLD isoformei dagokienez, PLD3, PLD4, PLD5 eta PLD6ek ez dute PH eta PX domeinurik beraien sekuentzietn. Bestalde, guztiek bi HKD domeinu dituzte, PLD6 izan ezik, HDK domeinu bakarra duenak[135].

PIP_2 (fosfatidilinositol 4,5-bifosfato) PLD-en jarduera katalitikoaren funtsezko kofaktorea da, eta berriki, PIP_2 PLD-ra lotzeko eremu bat aurkitu da. Eremu horren funtzioa zehazteko, Bruntz et al.-ek eremu hori mutatu zuten, PLD-ren jarduera katalitikoan beherakada nabarmena eraginez. Aldiz, PLDren kokapen azpizelularrean ez zuten aldaketarik aurkitu. Beraz, PIP_2-ren funtzioa alde batetik PLD mintzaren domeinu jakin batera errekrutatzea dela, eta bestetik substratua gune aktibora lotuz, PC-en katalisia estimulatzea izan daitekeela ondorioztatu zuten[134].

PLD-en funtzio ezagunena lipasa aktibitatea izan arren, entzima hauek proteina-proteina interakzioak ere burutu ditzazkete. PLD entzimen aktibazio katalitikoa bi bide desberdinetatik gerta daiteke. Alde batetik, tirosina kinasa hartzaileen (EGFR edo PDGFR) eta tirosina kinasa ez-hartzaileen (proteina kinasa c (PKC) , Src eta Janus kinasa 3 (JAK3)) bidezko fosforilazioak lipasa jarduera aktibatzen du[135]. Horrela, zelulaz kanpoko seinale mitogenikoek PLD-ren aktibazioa estimulatzen dute, horien artean kimokinak (IL-8), intsulina, hazkunde-faktoreak (hazkuntza faktore epidermikoa (EGF), plaketatik eratorritako hazkuntza faktorea (PDGF), endotelio baskularraren hazkuntza faktorea (VEGF)), gantz-azidoak eta esfingosina-1-fosfatoa (S1P) aurkitzen dira, besteak beste[136].

Bestalde, PLD2 isoformak guanina nukleotidoak trukatzeko faktorearen (GEF) jarduera dauka, zeinak zelulen mugikortasunean eragiten duen. PLD-en funtzio ezagunena lipasa aktibitatea izan arren, PLD2 entzimak proteina-proteina elkarrekintzak ere arautzen ditu. Horrela, PLD2ren aktibazioaren erregulazioa Rac2, Arf, Rho eta Ras bezalako GTPasa txikien bidez gauzatzen da, besteak beste. Gainera, ikusi da azido fosfatidikoak (PA) kontrolatzen duela PLD2-ren GEF jarduera, sofistikazio handiagoa gehituz entzimaren erregulazioari[135,137].

PLD2-ren aktibazioaren ondoren sortzen den PA molekula, liso-PA-ra eraldatu daiteke PLA_2-ren bitartez, edo DAG-ra PA-fosfohidrolasaren bidez. Beraz, PLD entzimek funtsezko garrantzia dute seinaleztapen zelularrean, sortutako molekulak bitartekari lipidiko garrantzitsuak baitira seinaleztapen zelularreko hainbat bideetan parte hartuz[133].

Izan ere, PA-ren ezaugarri garrantzitsuenetarako bat zelulen seinaleztapen bideetan parte hartzen duten proteina batzuekin dituen elkarrekintzak dira. Adibidez, mTOR, Akt eta Raf-1 (ERK seinaleztapen bideko kidea) proteinek PA-rekin elkarreragiten dute, eta horrek esaterako eragina du zelulen hazkuntzan eta biziraupenean[133].

Minbizian konkretuki, hainbat mekanismo transkripzionalek eta post-transkripzionalek entzimen adierazpena eta jarduera areagotzen laguntzen dute[138]. Horrela, hainbat neoplasiatan PLD1 eta PLD2-ren adierazpena eta jarduera handituta daude, esaterako, melanoman, bularreko, giltzurruneko, koloneko, urdaileko, burmuineko, eta tiroideko minbizietan[139]. PLD2 konkretuki, koloneko eta giltzurruneko minbizian pronostiko txar batekin korrelazionatu da[140]. PLD-en adierazpen eta jarduera altuek tumore-progresioan eta metastasian inplikatuta dauden hainbat seinaleztapen bideri eragiten diete. Seinale mitogenikoen (EGF, PDGF) eta onkogenen adierazpenaren (Ras, Raf, Src) ondorioz ematen den PLD-en jardueraren gorakada MAPK, Ras eta mTOR-ren aktibatzearekin lotu da[140,141]. Bestalde, HIF-ek PLD2-ren adierazpena handitzen duela ikusi da[142]. Gainera, PLD entzimak garrantzitsuak dira minbizi zelulek jasaten duten metabolismo eraldaketari eusteko. Zehazki minbizi-zelulen eskari biosintetiko eta bioenergetikoak asetzeko, izan ere, PLD entzimek glukosaren xurgapena handitzen dute. Horren ildotik, PLD entzimek tumorearekin eta mikroinguruneарekin interakzioan dihardute, zelulen hazkuntza eta angiogenesia bultzatuz[142].

PLD entzimek berebiziko eragina dute prozesu metastatikoetan. Lipasa-jardueraren ondorioz sortutako PA eta seinaleztapen-molekula desberdinekin duten elkarrekintzak (Grb2, Rac2, Wasp, S6K eta JAK3, besteak beste) funtsezkoak dira zelulen mugigarritasuna eragiteko. Hala ere, giltzurruneko tumore primario eta metastatikoetan PLD1-aren adierazpen mailak nabarmeki ez direla aldatzen ikusi da. Beraz, PLD1-ek prozesu metastatikoan laguntzen badu ere, PLD2-k tumore-zelulen barreiadura metastatikorako funtsezko zeregina izan lezakeela iradokitzen da[140]. Horrela, PLD1-ek mikroingurune tumoralean aritzen dela nagusiki, eta aldiz PLD2 tumore-zeluletan zuzenean eragiten duela proposatu da[145].

Bestetik, PLD2 batez ere prozesu metastatikoarekin erlazionatu da. PLD2-ren jarduerak hainbat minbizien hazkuntza eta inbasio ahalmena sustatzen ditu, hala nola linfoman[146] eta bularreko minbizian[139]. Gainera, PLD2-k FAK fosforilatuz eta Akt eta mTOR-ren aktibazioaren bidez prozesu metastatikoa eragiten duela frogatu da[146]. Izan ere, PLD2-k matrizearen degradazioa, inbadopodioen eraketa, tumore-zelulen migrazioa, inbasioa eta bularreko minbizi-zelulen barreiadura metastatikoa bultzatzen dituela deskribatu da. PLD2-k tumore zeluletatik ematen den metaloproteinasa jariaketa areagotu, eta aktina-zitoeskeletoaren berrantolaketa sustatzen du[147]. Bere inaktibazioak, aldiz, zelula hazkuntza, atxikidura, migrazioa eta inbasioa gutxitzen dituela ikusi da[146]. Bularreko minbizian, PLD2-ren gainadierazpenaren ondorioz, saguetan tumorearen hazkuntza handitu, kimioerresistentzia areagotu eta apoptosiaren murrizketa ikusi zen; PLD2-ren isilpena, berriz, nabarmenki gutxitu zituen zelula hazkuntza eta biriketako metastasiak[139].

HIPOTESIA ETA HELBURUAK

1. Hipotesia

Melanoma, melanozitoen eraldaketa gaiztoaren ondorioz sortzen den minbizia da. Toki ezberdinenetan sor daitekeen arren, normalean larruazalean agertzen da. Melanomaren intzidentzia etengabe handitu da azken hamarkadetan, eta Europan zazpigarren minbizi diagnostikatuena da. Larruazaleko tumore guztien %4a baino ez bada ere, minbizi horiek eragindako heriotzen %80ren erantzule da (street). Aipatzekoa da, 2019an, Espainian 150000 kasu berri diagnostikatuko direla iragarri dela (who).

Melanoma tumoreetan ikusitako heterogeneotasunak biomarkatzaileak edo benetan eraginkorrak diren tratamenduak identifikatzea zaildu du. Horregatik, heriotza tasak oso altuak dira, melanoma metastatikoarentzat bereziki. Beraz, itu terapeutiko gisa erabil daitezkeen edo detekzio goiztiarrean, diagnostikoan eta pronostikoan lagun dezaketen biomarkatzaile berriak identifikatzeko premia handia dago; izan ere, errekuperazio-tasak oso handiak dira tumorea goiz detektatzen bada.

Ildo horretan, zelulen metabolismoa minbiziaren ezaugarri gisa ezarri da. Jakina da zelula horiek jasaten dituzten bide metabolikoen aldaketak tumore zelulek hartutako fenotipo gaiztoa babesten dutela. Zehazki, lipidoen metabolismoak prozesu kartzinogenikoan duen inplikazioak interesa sortu du minbiziaren ikerketaarloan. Metabolismoa eraldatuta dago zelula tumoraletan, eta horrek lipido batzuen konposizioan eta kantitatean aldaketak eragiten ditu. Horregatik, hurrengo hipotesia planteatu da: lipidoen konposizio eta kantitatean eta horien metabolismoan gertatzen diren aldaketek, melanozito ez-patologikoak melanoman bihurtzea eragin dezakete. Beraz, melanozito ez patologikoekin alderatuz, melanoman modu aberrantean adierazten diren espezie lipidiko jakinen identifikazioa, gaixotasun honetarako biomarkatzaile garrantzitsuak aurkitzea izan liteke. Bestalde, espezie horiek melanomaren garapenean eta progresioan duten inplikazioa ezagutzea ahalbidetuko luke. Lipidoen edukian ematen diren aldakuntzak molekula horien metabolismoan parte hartzen duten entzimen adierazpen eta jarduera-alterazioekin batera doaz. Adibidez, fosfolipasa entzimek glizerofosfolipidoen katabolismoan hartzen dute parte, eta entzima familia horren isoforma ezberdinak minbiziarekin lotu izan dira. Izan ere, PLD2-k hainbat minbizi zelulen gaiztotze prozesuan eta barreiadura metastasikoan laguntzen duela frogatu da.

2. Helburuak

Doktorego tesi honen helburu nagusia melanomarentzako biomarkatzaile lipidiko berriak identifikatzea izan zen, melanomaren detekzio goiztiarrean, diagnostikoan eta pronostikoan lagunduko zutenak. Horretarako, hainbat ikuspegi lipidomiko aplikatu ziren. Gainera, lan honetan lortutako emaitzek melanomaren garapenean eta progresioan inplikatutako mekanismo molekular eta zelularrak hobeto ulertzen lagunduko zuela espero zen.

Horrela, doktorego tesi honen helburu nagusiak hurrengo helburu espezifikoetan zehaztu diren ziren:

- Melanozito osasuntsuen, nevusetatik eratorritako melanozitoen, melanoma primarioen eta melanoma metastatikoen lerro zelularren lipidoma aztertu, hainbat lerro zelularretatik erauzitako lipido erauzkinen analisi lipidomikoa eginez.
- Biomarkatzaile lipidomikoak detektatzeko erabili daitekeen translazio-potentziala duen tresna bioteknologiko eraginkor berri bat ezarri.
- Melanoma diagnostikatzeko eta pronostikoa definitzeko balioa duten espezie lipidiko zehatzak identifikatu.
- PLD2 entzimak melanomaren jarduera kartzinogenikoetan duen inplikazio funtzionalak zehaztu *in vitro*.

EZTABAIDA

1. Giza melanozito eta melanoma lerro zelular ezberdinen profil lipidiko globala

Larruazaleko melanomaren intzidentzia-tasa etengabe igo da azken hamarkadetan. Izan ere, Europan gehien diagnostikatutako zazpigarren minbizi mota da, eta larruazaleko minbiziek eragindako heriotzen %80ren erantzulea da[1]. Hala ere, heriotza-tasak ez du intzidentziaren joera bera jarraitu, eta egonkor mantendu da azken 20 urteetan. Egonkortze hori kontzientzian, diagnostiko goiztiarrean eta tratamenduetan izandako hobekuntzengatik gertatu da[4]. Aipatu beharra dago 2011z geroztik FDA-k 10 tratamendu-aukera desberdin onartu dituela, urtebeteko biziraupena orokorra %16-30retik %50-70rera handituz. Pazientearen etorkizunak zerikusi zuzena du diagnostikoa ematen denn etaparekin. Bada, errekuperazio-tasa %100 ingurukoa izaten da diagnostiko goiztiarra egiten denean, aldiz, ez dago terapia eraginkorrik melanoma metastatikoa tratatzeko[29]. Diagnostikoa behaketa klinikoan eta geroagoko baieztapen histopatologikoan oinarrituta egiten da. Hala ere, ez dago biomarkatzaile erabat eraginkorrik larruazaleko melanomarentzat. Beraz, diagnostiko eta pronostiko goiztiarrean lagun dezaketen edo itu terapeutikoak izan daitezkeen biomarkatzaile berriak aurkitzea premiazkoa suertatzen da.

Azkenaldian, zelulen eduki lipidikoaren, hau da lipidoma, eta lipido horien metabolismoaren azterketak minbiziarekin duen lotura etorkizun handiko ikerketa eremu bihurtu da. Izan ere, zelula-energiaren kudeaketaren desrregulazioa eta egokitzapen metabolikoa minbizi zelulek bereganatutako ezaugarriak dira[42]. Lan askok erakusten dute tumore zelulek beren lipido edukia eta horien metabolismoa egokitzen dituztela zelulen fenotipo gaiztoa bermatzeko. Aldaketa horiek tumorearen mikroingurumenak ezarritako egoera latzetan bizirautea, ugaritzea eta haztea ahalbidetzen dute. Tumore-zeluletan gertatzen den lipido-metabolismoaren egokitzapena, bereziki, lipidoen *de novo* biosintesi eta oxidazio areagotu batek definitzen du. Alterazio horiek seinaleztapen-bide aberranteak, energia ekoizpen etenduna, zelula-mintzetan egitura-alterazioak, eta gene eta proteinen bestelako adierazpena sortzen dituzte. Horren ondorioz, zelula-hazkuntza, ugalketa, heriotza desarautua eta farmakoekiko eta kimioterapiarekiko erresistentzia ematen da[49], besteak beste. Gainera, minbizi zelulen lipido metabolismo aberranteak zelulen barreiadura metastatikoarekin lotura estua duela frogatu da[114]. Hori bularreko, koloneko, biriketako eta prostatatako minbizietan aztertu da bereziki[49,96,159], baina ezer gutxi dakigu lipidoma eta metabolismo aldaketek melanoma zeluletan eragiten dituzten alterazioei buruz.

Horretarako, melanozitoen eta melanoma zelulen lipidomaren azterketa bereizgarri bat proposatu zen, melanoma eta melanozito zelulen aztarna lipidiko globala aztertzeko. Bestalde, zelula normal eta minbizidunek lipido eduki ezberdinak izatea azaltzen dituzten lipido espezie partikularrak identifikatu, eta aldaketa horien inplikazio biologikoa ere ikasi nahi zen. Horretarako, larruazaleko eta nevuseko melanozitoen lipidoma aztertu zen, bai eta melanoma primarioaren eta metastatikoen lerro zelularrena ere, hainbat metodologia lipidomiko erabiliz. Horiek tumore zelulak eta ehunak identifikatzeko eta sailkatzeko erabiltzen dira, lagin baten lipido edukia eta lipido horiek beste lipido batzuekin, proteinekin, metabolitoekin, eta eduki genetikoarekin dituzten elkarrekintzak definitzen dituzte[96].

Analisi lipidomikoak, nagusiki, masa espektrometria tekniketan oinarritzen dira. ESI (elektroesprai bidezko ionizazioa) eta MALDI (laser bidezko desortzioa eta ionizazioa matrize bidez lagunduta) masa espektrometria bidez biomolekulak aztertzeko ionizazio-iturri garrantzitsuenak dira (chen). MALDI-MS biomarkatzaileak identifikatzeko erabiltzen da maiz, eta lipidoak zuzenean identifikatzeko aplikatzen da, ez baitira lipidoak aurretik erauzi behar. MALDI-MS-ak pisu molekular txikiko matrize organiko baten metaketa behar du, laser erradiazioa

xurgatzen duenak eta ioi molekularrak igortzen dituenak. MALDI ionizazioa erabiltzearen abantaila nagusiak hainbat dira, hala nola molekulak markatzeko beharrik ez izatea, sentsibilitate handia izatea, errendimendu altuko teknika erabiltzea eta espezifikotasun molekularra izatea[105]. Hala ere, MALDI ionizazio leuneko teknika bat denez, lipidoaren egitura orokorraren informazio lortzen da soilik, alboko kateak zeintzuk diren zehaztu gabe. Hori gainditzeko, ESI-MS/MS tandem metodologia erabil daiteke. Hori sarritan kromatografia likidoarekin konbinatzen da lipido erauzkinetan dauden lipido familiak aldez aurretik bereizteko molekulen identifikazio zehatzago bat lortuz. Gainera, tandem bidezko MS/MS estrategia bat erabiltzean, ioi aitzindaria eta horren zatiketaren ondorioz sortzen diren molekulak detektatzen dira, molekula aitzindariaren egitura eratuz. Izan ere, ESI lipidoetarako gehien erabiltzen den ionizazio-metodoa da[105].

Lan honetan burutu den lehenengo estrategian larruazaleko melanozitoetatik, nevuseko melanozitoetatik, melanoma primarioetatik eta melanoma metastatikoetatik erauzitako estraktu lipidikoen edukian dauden ezberdintasunak identifikatu ziren. Horretarako, lipidoen azterketarako oso erabilia den UHPLC-ESI-MS/MS estrategia erabili zen. Lortutako emaitzak analisi estatistiko ez-gainbegiratuen (PCA) eta gainbegiratuen (PLS-DA) bidez aztertu ziren. Larruazaleko eta nevuseko melanozitoen, eta bestaldetik, melanoma primario eta metastatikoen edukia alderatzean, ez ziren ezberdintasun nabarmenak identifikatu ikuspegi analitiko horiek erabilita. Ostera, zelula osasuntsuek (larruazaleko eta nevuseko melanozitoek) eta gaiztoek (melanoma primario eta metastatikoek) lipido profil ezberdina dutela ziurtatu zen. Beraz, erabilitako ikuspegi lipidomikoa egokia da minbizi zelulek erakusten duten lipidoma aberrantea aztertzeko.

Emaitzen ikuspegi sakonago batek agerian utzi zuen lipido-familia bakoitzean lortutako intentsitateak azterketa-taldeen arabera aldatzen direla, lipidoen edukiaren aldaketek eraldaketa gaiztoari eta minbizi-zelulen biziraupenari lagunduz. Gure emaitzen deskribapen orokor batek, kolinadun lipidoek melanoma metastatiko zeluletan azaleko melanozitoetan baino intentsitate altuagoa erakusten dutela plazaratzen du. Ezaugarri hori hainbat tumoreetan ikusi da, hala nola, bularreko, garuneko, biriketako, obario eta prostatako minbizietan[153,154]. Kolina glizerolipidoek egitura-funtzioa betetzen dute bereziki, mintzaren lipidoen %50 baitira. Honetaz gain, seinaleztapen molekulak ere badira, seinale mitogenikoetan bihurtzen baitira. Familia horretan antzemandako intentsitate altuak minbizi zelulek jasaten dituzten metabolismo aldaketekin lotu izan dira. Lehenik, kolinaren xurgapena handitzea minbizi ezberdinen ezaugarri komuna da, eta, horretarako, kolina-garraiatzaileak behar dira, melanoma zeluletan oso gainadieraziak aurkitu direnak[127]. Biosintesiari dagokionez, Kennedy bideko lehen entzima, kolina kinasa, modu aberrantean detektatu da biriketako, koloneko, bularreko, prostatako, umetoki-lepoko eta obarioko tumore zeluletan. Kolinaren sintesian ematen diren aldaketak, Ras eta PI3K seinaleztapen bidean ikusi diren alterazioen ondorioz sortzen direla proposatu da[55]. Bide horiek melanomaren bide nagusiak dira eta modu konstitutiboan aktibatzen dira. Modu nabarmenean, kolina-lipidoen intentsitateak kimioterapiaren eraginkortasuna kontrolatzeko erabiltzen dira, seinale gutxituak erantzun terapeutiko on bati lotzen baitira[123]. Aitzitik, gure azterlanean hautemandako mintzetan kokatzen diren bigarren glizerofosfolipo familia ugarienak fosfatidiletanolamina duten espezie lipidikoak izan dira. Horien intentsitatea murriztuta identifikatu da melanoma zeluletan eta nevuseko melanozitoetan, larruazaleko melanozitoekin alderatuta. Hala ere, aldakuntza handia detektatu da azterketa talde bereko zelula lerroen mailetan. Izan ere, bibliografian eztabaida handia dago minbizi zeluletan fosfatiletanolamina lipidoek dituzten adierazpen mailekin, eta emaitzak minbizi motaren araberakoak dira. Adibidez, gure emaitzen antzera, PE mailak gutxituta agertu

ziren giltzurrun kartzinoman, eta, zelula gaizto horiek etanolaminarekin inkubatzean, zelulen hazkuntza murriztu egin zen, zelula normaletan inolako eraginik detektatu ez zen bitartean[155].

Mintzen homeostasia, bere konposizio lipidikoaren araberakoa da, eta bere eduki lipidikoan aldaketa txikiek ere mintzaren integritatea aldatzen dute. Fosfolipidoek mintz lipido gehienak hartzen dituzte, eta horien buru-polarrak zein azilo albo-kateek mintzen propietate biofisikoak zehazten dituzte. PC-ak, bigeruzetan gehien agertzen diren lipidoak, forma molekular zilindriko bat dute, euren buru taldearen tamaina dela eta. Horri esker, estuki lotzen dira, mintz egonkor bat osatuz. Gainera, PC-ek normalean cis-asegabea den gantz azido kate bat izaten dute, eta horrek fosfolipidoen paketatzea murrizten du eta mintzaren jariakortasuna handituz. Aldiz, PE-k forma konikoa dute, beren buru-polarreko taldearen tamaina txikia dela eta, eta horrek tentsio negatiboko kurbadura bat ezartzen du mintzean. Horrek lipidoen paketatze-akatsak sortzen ditu eta mintzaren fusioa, fisioa eta gemazioa errazten du[156,157]. Beraz, lipido zilindriko/konikoen proportzioa kritikoa da bigeruzen osotasun eta funtzio egokiari eusteko; izan ere, PCak kanpoko xaflan daude nagusiki, eta PE gehienak, berriz, xafla zitoplasmatikoan[156,158]. Gure lanean lortutako PC/PE proportzioek erakusten dute, ratio hori areagotu egiten dela melanomaren garapenarekin; izan ere, azaleko melanozitoetan 1ekoa da, eta 1,5era igotzen da melanoma primarioan eta 1,7ra melanoma metastatikoan. Horrek esan nahi du melanoma zelulen mintzen jarioa eta iragazkortasuna handituta dagoela[159,160]. Ezaugarri hori tumore solido askotan ikusten da, fenotipo ugalkor eta inbaditzailea sustatzen baitu. Izan ere, tumore zelulen potentzial metastatikoa nabarmen korrelatzen da mintzaren jariakortasun areagotuarekin[161]. Gainera, tratamendu kimioterapeutiko eraginkorrak mintzaren jariakortasuna murriztearekin eta mintzaren iragazkortasuna handitzearekin lotu dira; aldiz, zelula erresistenteek mintz zurruna, eta, beraz, iragazkortasun txikiagoko mintza erakusten dute, farmakoaren sarrera eragotziz[162]. Minbizi mota askotan ikusitako kolina lipidoen xurgapen eta *de novo* sintesi areagotuak ez lirateke nahikoak izango hazkuntza tasa oso altua duten minbizi zelulek behar duten PC guztia hornitzeko. Horretarako, baliteke melanoma zelulek PEMT-ren adierazpena eta jarduera areagotzea, euren kolina beharrak asetzeko. PEMT mintzeko entzima bat da, PE-ak zuzenean PC bihurtzen dituena ondoz ondoko hiru metilazioren ondoren. Beste tumore mota batzuei dagokienez, datuak ez dira sendoak; gibeleko eta bularreko minbizian haien jarduera nabarmen jaisten bada ere, gerora etanolamina-lipidoen presentzia areagotuz[127], kontrako emaitzak ikusi dira biriketako minbizi zeluletan, gure emaitzekin bat eginez[153]. Oro har, tumore erasokorrak PC/PE erlazioa handitzearekin eta PEMT jarduera handiagoarekin lotu dira[153]. Gure emaitzek entzima horrek melanomaren garapenean jarduera handiagoa izan dezakeela iradokitzen dute; izan ere, azilo-kate berberak partekatzen dituzten PC/PE lipido espezie zehatzen arteko proportzioa handitu egiten da melanoma metastatikoan kasu guztietan bat izan ezik: PC(16:0/20:4) - PE(18:1/18:3) edo PE(16:0/20:4).

Taula 4. Azilo albo-kate berdinak dituzten PC eta PE espezieen intentsitatearen PC/PE proportzioa, kontroleko taldeak lortutako emaitzekin erlazionatuta (larruazaleko melanozitoak = 1).

	PC/PE proportzioa larruazaleko melanozitoekin erlazionatuta			
	Larruazaleko melanozitoak	Nevuseko melanozitoak	Melanoma primarioa	Melanoma metastatikoa
PC(16:0/16:1)/ PC(14:0/18:1)	1,00	1,09	1,61	2,26
PE(16:0/16:1)				
PC(16:0/18:2)	1,00	2,39	0,73	1,32
PE(34:2)				
PC(16:0/18:1)	1,00	1,32	1,43	1,65
PE(34:1)				
PC(18:0/18:1)	1,00	2,46	1,26	2,10
PE(18:0/18:1)				
PC(38:3)	1,00	2,12	1,62	2,25
PE(18:1/20:2)/ PE(18:0/20:3)				
PC(18:0/20:2)	1,00	1,28	3,26	4,33
PE(18:1/20:1)/ PE(18:0/20:2)				
PC(40:5)	1,00	1,97	1,05	1,23
PE(18:1/22:4)/ PE(20:2/20:3)				
PC(40:4)	1,00	2,42	2,07	1,76
PE(18:0/22:4)				
PC(16:0/20:4)	1,00	1,50	0,88	0,87
PE(18:1/18:3)/ PE(16:0/20:4)				

Kolina eta etanolamina eter lipidoak mintzetan dauden fosfolipido guztien %15-20 inguru dira, eta egitura-eginkizun garrantzitsua betetzen dute hemen. Gure emaitzen ikuspegi orokor batek melanoma lerro zelularretan kolina eter lipidoen intentsitatea nabarmen handitzen dela erakusten du. Aitzitik, etanolamina eter lipidoak lerro zelular gaiztoetan murrizten dira, eta hori, alde batetik, bularreko minbizi zelula migratzaileak eta epitelio zelulak[163], eta bestetik, kolon adenokartzinoma zelulak eta ehun normaleko zelulak[164] konparatzen zituzten beste lan batzuetan lortutako emaitzekin korrelazionatzen du. Izan ere, eter lipidoen presentzia handitua fenotipo ugaltzailearekin eta minbizi-zelulen potentzial tumorigenikoarekin erlazionatu da[125]. Eter lipidoen biosintesiaren lehen urratsak peroxisometan egiten dira, AGPS (alkilglizerona fosfato sintasa) bidearen lehen urratsaren arduraduna delarik. Entzima hori melanoma metastatikoan, bularreko eta prostatako minbizietan gainadierazten da. Bere inaktibazioa ostera, eter lipidoen sintesia murriztu, eta melanoma eta bularreko minbizi zelulen mugigarritasuna eta inbasio ahalmena gutxitzen ditu[72]. Gure lanean eter lipidoetan lortutako emaitzek kolina eta etanolamina glizerofosfolipidoen joera bera jarraitzen dute, eta horrek melanoma zeluletan kolina molekula gehiago dagoela eta etanolamina talde nagusiaren presentzia eskasa dagoela iradokitzen du. Minbizi zeluletan hautemandako ezaugarri komun bat PC/kolina eter lipidoen proportzioaren murrizketa da, gure emaitzetan ere agertzen dena; izan ere, melanoma primario eta metastatikoek PC/kolina erlazioan jaitsiera nabarmena erakusten dute (0,3), azaleko melanozitoekin alderatuta (1). Nevus melanozitoek aitzitik, PC/kolina eter

lipidoen proportzioan igoera nabarmena erakusten dute (2,9), larruazaleko melanozitoekin alderatuta (1). Emaitza horien arabera, baliteke peroxisomak nevus eta melanomaren garapenean eta progresioan parte hartzea. Aldiz, etanolamina lipidoetarako ez zen desberdintasun nabarmenik ikusi, ez melanoman, ezta nevus zeluletan ere.

Presentzia erlatibo txikiagoa izan arren, glizerofosfolipido anionikoak, fosfatidilserina, fosfatidilinositola eta fosfatidilglizerola barne, batez ere bigeruzen aurpegi zitoplasmatikoan agertzen dira, eta egitura-funtzio garrantzitsua dute[158]. Izan ere, mintzen gainazaleko karga zehazten laguntzen dute eta, ondorioz, positiboki kargatuta dauden mintzeko proteinekin edo proteina periferikoekin elkarrekintzak modulatzen dituzte[156]. Fosfatidilserinek konkretuki, zelula tumoraletan oso garrantzitsuak diren seinaleztapen-molekulekin elkarreragiten dute. Adibidez, PS Ras eta Rho familiako GTP-asekin lotzen da, baita Src kinasa tirosinarekin ere, bere mintzerako mobilizazioa modulatuz eta ondoren aktibatuz[57]. Gainera, PS Akt-ra lotzen da, eta horrek Akt-ren seinaleztapen bidea aktibatzen du, zelulen biziraupena areagotuz[165]. PS-ren biosintesia ugaztun zeluletan, buru polarrak trukatzeko erreakzio baten bidez gauzatzen da, jada existitzen den PC edo PE baten talde nagusia L-serinarekin ordezkatzen baita. Erreakzio hori PSS1-ek katalizatzen du (fosfatidilserina sintasa 1), baldin eta trukea PC-tik egiten bada, eta PSS2-k etanolaminaren ordezkapenerako jarduten du[57]. Gure emaitzek PSS1 melanoma primario eta metastatiko zeluletan inhibituta egon daitekeela iradokitzen dute; izan ere, badirudi azilo-kate berberak dituzten PC-PS molekulen bihurketa gutxitu egiten dela zelula gaiztoetan (5. taula). Hala ere, ez da aldaketa esanguratsurik ikusi PE-PS bihurketan; hortaz, ez da aldaketarik espero PSS2-ren jardueran. Bestalde, PS-ren funtzio garrantzitsu bat zelula barruan PE sintetizatzea da, PS deskarboxilasa entzima mitokondrialaren bidez (PSD), zebra-arraina melanoma ereduan gainadierazia agertzen dena[132]. Izan ere, gure emaitzetan ikus dezakegu azilo albo-kate berdinak dituzten PE eta PS molekulen kasuan, horien arteko erlazioa handiagoa dena 3 kasuetatik baten. Beste bi kasuetan ostera, igoera txikia da (6. taula). Laburbilduz, gure emaitzek zelula gaiztoetan ematen den PC metaketa azaltzen laguntzen dute, PSS1 bidez ematen den PC-PS bihurketa negatiboki araututa dagoela baitirudi. Gainera, gure zelula tumoraletan PE maila baxuak daudenez, PSS2 bidez PE-PS bihurketa inhibitu egin liteke, eta PE ekoizteko PS-ren deskarboxilazioa melanoman apurtxo bat handituta dago, baina molekula gutxi daude, eta, beraz, ikerketa gehiago behar da ondorio esanguratsu batera iristeko.

Taula 5. Azilo albo-kate berdinak dituzten PC eta PS espezieen intentsitatearen PC/PS proportzioa, kontroleko taldeak lortutako emaitzekin erlazionatuta (larruazaleko melanozitoak = 1).

	PC/PS proportzioa larruazaleko melanozitoekin erlazionatuta			
	Larruazaleko melanozitoak	Nevuseko melanozitoak	Melanoma primarioa	Melanoma metastatikoa
PC(16:0/18:1)	1,00	0,98	1,50	1,63
PS(16:0/18:1)				
PC(18:1/18:1)/PC(18:0/18:2)	1,00	1,54	1,82	2,57
PS(18:2/18:0)/PS(18:0/18:2)/PS(18:1/18:1)				
PC(18:0/18:1)	1,00	2,04	2,21	3,98
PS(18:1/18:0)/PS(18:0/18:1)/PS(16:0/20:1)				
PC(18:0/20:2)	1,00	1,04	3,89	4,63
PS(18:0/20:2)/PS(18:1/20:1)				
PC(40:2)	1,00	0,90	1,61	2,23
PS(18:1/22:1)/PS(18:0/22:2)				

Taula 6. Azilo albo-kate berdinak dituzten PE eta PS espezieen intentsitatearen PE/PS proportzioa, kontroleko taldeak lortutako emaitzekin erlazionatuta (larruazaleko melanozitoak = 1).

	PE/PS proportzioa larruazaleko melanozitoekin erlazionatuta			
	Larruazaleko melanozitoak	Nevuseko melanozitoak	Melanoma primarioak	Melanoma metastatikoak
PE(34:1)	1,00	0,74	1,05	0,99
PS(16:0/18:1)				
PE(18:0/18:1)	1,00	0,83	1,76	1,89
PS(18:1/18:0)/PS(18:0/18:1)/ PS(16:0/20:1)				
PE(18:1/20:1)/PE(18:0/20:2)	1,00	0,81	1,19	1,07
PS(18:0/20:2)/PS(18:1/20:1)				

Gure azterketan, fosfatidilinositolak, zelula-mintzetan kokatzen den glizerofosfolipido anionikoen beste azpimota bat, melanoma metastatikoetan euren intentsitatea areagotzen dutela ikusi da. Ezaugarri hori bera beste autore batzuk ere antzeman zuten melanoma metastatikoan[166]. PI zikloan, PI3K-k zuzendutako PI-ren fosforilazio itzulgarria dago. Entzima hori minbizi mota askotan mutatuta aurkitu da, melanoman barne. Entzima horren produktua, PIP_3, bigarren mezulari pro-tumoral indartsu bat da, ahalmen metastatikoarekin estuki lotu dena, eta ugalketa, hazkuntza eta zelulen migrazioa modulatzen baititu[166]. Hala ere, gure lanean erabili dien analisi lipidomikoekin ezin dira PI fosforilatuak ikasi, eta, beraz, soilik PI mailak gora egin duela baieztatu dezakegu.

Mintzetan aurkitzen den lipido anionikoen azken azpimota fosfatidilglizerolak dira, nahiz eta kopuru oso baxuetan dauden (glizerofosfolipido guztien %1-2)[65]. Gure emaitzek larruazaleko melanozito (1,00) eta melanoma metastatiko (1,02) zelula lerroetan PG mailak ez direla aldatzen erakusten dute. Hala ere, nevuseko melanozitoen (0,65) eta melanoma primarioen (0,54) mailak murriztuak aurkitu dira. PG-ak kardiolipina molekulen aitzindariak dira, mitokondrio-funtzio egokia mantentzeko garrantzitsuak direnak. Hala ere, ez dugu kardiolipinarik detektatu gure lanean, horien masa molekularra 1500 unitate baino gehiagokoa baita, eta erabilitako masa-espektrometria estrategiarekin, 50 eta 1200 unitate arteko masadun espezie molekular lipidikoak bakarrik detektatu ditugulako.

Zelula-mintzetan glizerofosfolipidoekin batera dagoen beste lipido mota garrantzitsu bat, esfingolipidoak dira. GPL-ek ez bezala, esfingolipidoek, nagusiki, albo-kateetan gantz-azido aseak edo trans-asegabeak dituzte, zilindro-formako egitura altuago eta estuago bat osatuz. Horrela mintzaren paketatze-dentsitatea handiagoa da eta bigeruzaren barruko mugikortasuna aldiz, txikiagoa. Zeramiden egitura zurrunak ere bigeruzen arintasuna gutxitzen parte hartzen du[156,157]. Esfingomielina, ugaztun-mintzetan ugariena den esfingolipidoen azpiklasea da[158], eta kolina molekula bat du bere egituran. Gure emaitzetan, esfingomielinen eta zeramiden intentsitatean murrizketa nabarmena aurkitu dugu zelula gaiztoetan. Beraz, horrek tumore zelulen bigeruzen jariakortasuna handitzen laguntzen duela iradokitzen dugu, eta hori bat dator kolina-lipidoen gorakadarekin eta kolina/etanolamina lipidoen erlazioan lortutako emaitzekin. Izan ere, ezaugarri horrek zelula gaiztoen mugikortasuna eta ahalmen metastatikoa sustatzen ditu, deformazio ahalmen handiagoa baitute[145,161,167]. Edmond et al-ek epitelio-mesenkima trantsizioek mintzaren jariakortasuna handitzeko esfingolipidoen mailak aldatzen dituztela eta, horrela, zelulen migrazioa errazten dutela proposatu zuten[168].

Esfingomielina espezieen maila baxuak partzialki azal daitezke, SM-en sintesi murriztua dela eta. Izan ere, esfingomielina sintasa 1 (SMS1) hainbat minbizi ehunetan modu negatiboan

erregulatuta aurkitu da[166,169]. Zehazki, melanoman, melanoma primarioan zein metastatikoan larruazaleko melanozitoekin eta nevusekin alderatuta SMS1 mailak jaisten direla frogatu da. Gainera, ezaugarri bereizgarri hori melanoma metastatikoa duten pazienteek emaitza okerragoa izatearekin eta melanomaren zelulen migrazio ahalmen handiagoarekin lotu da. Horrek, SM-en maila baxuek melanomaren progresioan parte hartzen dutela iradokitzen du[166,169]. Sintesia murriztuta egoteaz gain, SM-en metabolismoaren gorakadak ere lagun lezake zelula gaiztoetan aurkitu ditugun SM maila baxuetan. SM-en katabolismoaren ondoren, zeramida bat sortzen da. Azilo-kate berdinak dituzten esfingomielina/zeramida espezieen proportzioak behera egiten du melanoman, hau da, zeramiden intentsitateak gora egiten du dagokion esfingomielinarekin alderatuta. Horrek, bihurketa hori gure melanoma lerro zelularretan handituta dagoela iradokitzen du (7. taula). Aldiz, beste egile batzuk zeramidak sortzen dituzten entzimak, hau da esfingomielinasak, melanoman gutxituta daudela zehaztu dute[170–172]. Horren ondorioz SM-Cer bihurketak behera egingo luke. Beste egile batzuek ostera, giltzurrun kartzinoman bihurketa hori handituta dagoela ikusi zuten, guk lortutako emaitzekin bat etorriz[155]. Gainera, esfingomielinasaren jardueraren ondorioz, fosfokolina bat ere askatzen da, kolina-lipidoen sintesia areagotzeko erabil daitekeena eta, beraz, gure aurreko emaitzekin bat datorrena.

Taula 7. Azilo albo-kate berdina duten SM eta Cer espezieen intentsitatearen SM/Cer proportzioa, kontroleko taldeak lortutako emaitzekin erlazionatuta (larruazaleko melanozitoak = 1).

	SM/Cer proportzioa larruazaleko melanozitoekin erlazionatuta			
	Larruazaleko melanozitoak	Nevuseko melanozitoak	Melanoma primarioak	Melanoma metastatikoak
SM(d18:1/16:0)	1,00	1,44	0,46	0,53
Cer(d18:1/16:0)				
SM(d18:1/18:0)	1,00	0,79	0,48	0,36
Cer(d18:1/18:0)				
SM(d16:1/24:1)/SM(d18:1/22:1)/ SM(d18:2/22:0)	1,00	0,93	0,39	0,44
Cer(d18:2/22:0)				
SM(d18:1/22:0)	1,00	1,12	0,35	0,39
Cer(d18:1/22:0)/Cer(d16:1/24:0)				
SM(d18:1/24:1)	1,00	0,95	0,36	0,36
Cer(d18:1/24:1)				
SM(d18:1/24:0)	1,00	1,67	0,51	0,55
Cer(d18:1/24:0)				

Fosfokolinarekin batera, zeramida ere sortzen da esfingomielinaren metabolismoaren ondoren. Zeramidak lipido bioaktibo oso garrantzitsuak dira, eta, gure lanean, melanoma zelulek zeramida maila oso txikiak erakutsi dituzte. Uchidaren taldeak deskribatu zuenez, melanozitoen zelula-kultiboei zeramida exogenoa gehitzeak melanozitoen hazkuntza oztopatzen du Akt-ren inaktibazioaren ondorioz[173]. Zeramidak molekula ez-tumorigenikotzat hartzen dira, zeramida-maila altuak hazkuntza gelditzearekin, seneszentziarekin, apoptosiarekin eta autofagiarekin erlazionatu baitira[174,175]. Gainera, zeramidek tumore-zelulen potentzial metastatikoa gutxitzen dute, integrinen azaleko adierazpena erregulatzen baitute[175]. Izan ere, tratamendu kimioterapeutiko batzuk, daunorrubizina, kanptotezina eta etoposidoa barne, zeramida mailak igotzen dituzte zelulen barruan, apoptosia eraginez[84,176]. Hala ere, tumore-zelulek zeramiden metabolismoa areagotzen dute funtzio pro-tumoralak dituzten beste esfingolipido batzuk sortuz. Adibidez, jakina da zeramidasa azidoa (ACDase) melanoman eta beste tumore zelula

batzuetan gainadierazten dela, eta zeramidak esfingosina-1-fosfato bihurtzea errazten duela. Molekula horrek melanoma zeluletan melanozito normaletan baino maila altuagoak erakusten ditu, eta tumore zelulen hazkuntza eta migrazioa sustatzen ditu[174,175]. Gainera, dakarbazinarekin *in vitro* egindako azterketek, terapiari erantzuten dioten lerro zelularretan, ACDase mailen dosi- eta denboraren-menpeko beherakada ematen dela erakutsi da; izan ere, lisosometan ACDase degradatu egin da eta zelula barneko 16-18 karbonodun zeramiden mailak areagotu egin dira[174,175,177]. Bitxia bada ere, ACDase-n inhibizio farmakologikoak zeramida mailak handitzen ditu, eta horrek eragile kimioterapeutikoen eta erradioterapiaren efektu zitotoxikoak sinergikoki hobetzen ditu melanoma kultiboetan. Gainera, ACDase-n espresio altua izateak melanomaren tratamenduarekiko erresistentziarekin erlazionatu da[84,174,176]. Gure emaitzei dagokienez, melanoma primarioek melanoma metastatikoek baino zeramida maila baxuagoak erakutsi dituzte, eta hori bat dator Realini *et al.* eta Leclerc *et al.*-en aurkikuntzekin. Lan horietan, hazkuntza-fasean dauden melanomek zelula inbaditzaileek baino ACDase maila eta aktibitate altuagoak erakusten dituztela frogatu zuten[174,178].

Melanoma zeluletan aurkitutako zeramida maila baxuak, bere katabolismoa handituta egon ez ezik, zeramida sintasa entzimen (CerS) bidez ematen den *de novo* sintesiaren gutxitzearen ondorio ere izan daiteke. Entzima horien sei isoforma ezberdin identifikatu dira, eta horietako bakoitzak funtzio ezberdinak betetzen dituzte ehunetan, kate luzera ezberdina duten zeramidak ekoizten baitituzte. Adibidez, CerS1-ek 18-karbonodun zeramidak sortzen ditu, apoptosiaren aldeko funtzioak dituztenak. Horren mailak gutxituta ikusi dira buru eta lepoko minbizi zeluletan, zeramida horien mailak murriztuz[84,179]. CerS6-k ostera, 16-karbonodun zeramidak ekoizten ditu, eta melanoman eta beste minbizi-ehun batzuetan negatiboki erregulatuta aurkitu da[84,175,179]. Izan ere, melanoma zeluletan CerS6 isilarazteak zelula horien hazkuntza eta inbasio-ahalmen handiagoa sortzen du, beste aldaketa batzuen artean[180]. Gure emaitzek melanoma zeluletan bai CerS1 bai CerS6 ere modu negatiboan araututa egon litezkeela iradokitzen dute, kate laburreko (16- eta 18-karbonodun) zeramida mailak txikiagoak baitira zelula gaiztoetan, azaleko melanozitoekin alderatuta. Hala ere, kate luzeko zeramidek (24-K) nevus eta melanoma garapenean beren mailak igotzen dituzte. Kate luzeko zeramidak CerS2-k sintetizatzen ditu, gure emaitza lipidomikoen arabera melanoma eta nevuseko zeluletan gainadierazita ager daitekeena. Izan ere, 24-K zeramidak zelulen biziraupenarekin erlazionatu dira[175,181].

Egitura-funtzioa ez duten lipidoei dagokienez, hala nola, gantz-azido askeak, diglizeridoak eta triglizeridoak, gure analisietanintentsitate baxua erakusten dute zelula gaiztoetan, bereziki triglizerido edukiari dagokionez melanoma metastatikoetan. Haatik, tumore ehunetan lipido klase horien maila altuak deskribatu dira batik bat, eta, beraz, ikerketa gehiago behar da hiru lipido familia horiek melanoman duten presentzia zehazteko.

2. Melanomarako biomarkatzaile lipidiko berrien aurkikuntza

UHPLC-MS/MS-n lortutako emaitza lipidomiko orokorrek lipido edukia melanozitoen eta melanoma zelulen artean aldatzen dela baieztatu zuten. Bestalde, lipido espezie bakoitzak azterketa talde bakoitzean izandako intentsitateen azterketak OPLS-DA analisi gainbegiratua erabiliz, argi utzi zuen 45 lipidoz osatutako panel bat dagoela melanozito eta melanoma zelulak bereiz ditzakeena. Emaitza horiek gure hipotesia baieztatu zuten: lipido espezie zehatzen presentzia larruazaleko melanozito arrunten eta melanomaren etapa desberdinen artean aldatzen da. Beraz, gure ikerketa melanoma-biomarkatzaileen aurkikuntzara bideratu genuen, translazio-potentziala duen tresna bat erabiliz: zelula-mintz funtzionalez osatutako mikroarraiak. Horietan, aztertutako lerro zelularretatik erauzitako mintzak immobilizatu ziren, eta MALDI-MS ikuspegi lipidomikoa aplikatu zen. Tresna hori onuragarria da hainbat arrazoiengatik: lagin bakoitzaren kantitate txiki bat nahikoa da, lipidoak erauzteko metodo konplexurik ez da behar, MALDI-MS biomarkatzaileak identifikatzeko metodo estandarra da, eta UHPLC-ESI-MS/MS-n antzemandako aldaketa garrantzitsu gehienak mintzean kokatzen diren lipido familietan ikusi ziren. Aldiz, MALDI-MS-aren eragozpen nagusietako bat ionizazio leuneko teknika lipidomiko bat dela da, eta, beraz, batzuetan ezin da molekularen azilo albo-kateen deskribapen osoa eman. Hori gainditzeko, bai MALDI-MS eta bai UHPLC-ESI-MS/MS bidez identifikatutako molekuletan, bigarrenarekin lortutako molekularen deskribapena erabiliz izendatu dira.

Lipido biomarkatzaileak aurkitzeko lehen estrategia t-test proba ($p<0.05$) bat egitea izan zen. Horretan, zelula ez-gaiztoetarako (larruazaleko + nevusetako melanozitoak) eta zelula gaiztoetarako (melanoma primario + metastatikoa) lortutako lipido espezie ezberdinen intentsitateak alderatuziren. 116 lipido espezie nabarmen aldatuta agertu ziren eta, beraz, biomarkatzaile lipidomikoak detektatzeko ikuspegi analitiko hori erabil genezakeela baieztatu genuen. Ondoren, konparazio anitzeko t-test proba bat eta post-hoc zuzenketak ($p<0.05$) erabili ziren detektatutako lipido espezieen intentsitatea lau azterketa-taldeen artean konparatzeko. Gure analisi estatistikoek lau azterketa-taldeen artean intentsitate mailak esanguratsuki aldatzen dituzten 122 lipido espezie daudela erakutsi zuten (8. taula).

Burututako PCA eta klusterizazio analisiek nevuseko melanozitoen lipidoma, azaleko melanozitoen antzekoa dela, eta aldiz, melanoma zelulena baino ezberdinagoa dela erakutsi zuten. Hori dela eta, nevuseko melanozitoak zelula ez gaizto gisa sailkatu ditugu burututako hainbat analisi estatistikotan. Hala ere, nagusiki neoplasia onberak diren arren, tumoreak dira, eta, beraz, euren lipidoma azaleko melanozito arruntetatik ezberdina da. Izan ere, azalaren eta nevuseko melanozitoen artean bereiztu dezaketen 11 lipido espezie aurkitu ditugu (8. taula). Bitxia bada ere, markatzaile horietako 10 azaleko melanozitoen eta melanoma primario edo metastatikoen zelulen arteko alderaketetan ere agertu dira. Horrek, molekula horien maila aldatuek nevusetik melanomarako trantsizioan lagundu dezaketela iradokitzen du. Bestalde, larruazaleko edo nevuseko melanozitoetan melanoma primarioetan lortutako emaitzak alderatzean, adierazpena nabarmenki aldatzen duten 48 eta 54 lipido espezie daudela ikusi zen. Gainera, nevus *versus* melanoma primarioko zelulen konparazioan esanguratsuak aurkitu ziren 54 lipido espezietatik 33 ere esanguratsuak izan ziren larruazaleko melanozitoak eta melanoma primarioak konparatzean. Beraz, 21 lipido espezie daude, euren mailak soilik nevuseko melanozitoetatik melanoma primariorako trantsizioan aldatzen dituztenak, eta, beraz, nevusetik abiatuta melanomaren garapenean markatzaile bezala erabil daitezkenak.

Larruazaleko edo nevuseko melanozitoen eta melanoma metastatikoen arteko lipido edukiaren aldea ere agerikoa da. Izan ere, larruazaleko edo nevuseko melanozitoen eta melanoma

metastatikoaren arteko biomarkatzaile potentzialak diren 82 eta 81 lipido espezie baitaude, hurrenez hurren (8. taula).

Melanomaren ikerketaren erronka nagusietako bat melanoma metastatikoarentzat progresio markatzaileak aurkitzea da, gaixoen biziraupen tasa izugarri jaisten baita tumorea goiz detektatzen ez bada. Era interesgarri batean, hiru lipido espezie daude, melanoma primariotik metastatikora euren adierazpena nabarmen handitzen dituztenak: 10 lipidoa, 11 lipidoa eta 22 lipidoa. Beraz, lipido espezie zehatz horien ikerketa sakonagoa egin behar da, melanoma primariotik metastatikorako progresioan duten zeregina ebaluatzeko.

Lipido erauzkinetan lortutako emaitzen joera bera jarraituz, 17 esfingomielinek bere intentsitatea nabarmen murriztu zuten zelula gaiztoetan, eta horrek minbizi zeluletan SM molekulen galera baieztatzen du. Gainera, bi DG molekula eta HexCer molekula bat zeuden melanozitoetan maila handituak zituztenak melanomarekin alderatuz. Bestalde, 13 PI molekulek euren intentsitatea esanguratsuki igotzen dute melanoman, melanoma metastatikoan bereziki. Lehen aipatu bezala, PI molekulak funtzio pro-tumoralak dituzte eta oso erlazionatuta daude minbizi zelulen potentzial metastatikoarekin. Beste lipido azpimoten barruan, lipido espezie guztiek ez dute joera bera jarraitzen. Adibidez, peroxisometan eratzen diren eter lipidoetan aldakortasuna dago, eta minbizian alteratuta daudela frogatu da. Horien artean, 18 PC eter lipido daude, horietatik 16k melanoma zeluletan euren mailak handitzen dituzte, eta bik, berriz, euren intentsitatea murriztu. Bestetik, maila diskriminatzaileak dituzten sei PE eter lipidoetatik 3 molekulek euren mailak igotzen dituzte melanoman, eta aldiz, beste hirurek murriztu. Gainera, PC edo PE eter lipidoak diren baieztatu ezin dugun arren, zazpi PC edo PE eter lipido esanguratsu daude eta horietako seik melanoman maila handitua dituzte. Emaitza horiek eta UHPLC-ESI-MS/MS atalean lortutakoek peroxisomek melanomaren garapenean eta progresioan zeregin garrantzitsua betetzen dutela baieztatzen dute, eta, beraz, organulu horien azterketa zehatzagoa behar dela, horien ekarpena ulertzeko.

Lipido espezie ezberdinen mailetan ikusten den aldakortasunak molekula bakoitzak eginkizun bakarra duela erakusten du. Molekularen buru polarrak eta azilo albo-kateen konposizioa molekula bakoitzaren funtzioa zehazteko oso garrantzitsuak dira. Beraz, lan honetan atzemandako biomarkatzaile potentzial guztien papera banan-banan ikertzea interesgarria izango lirateke. Bestalde, melanoma zelulen eta melanozitoen lipidoma ezberdina dela baieztatu ondoren, analisi lipidomiko ezberdinak eta osagarriak egin behar dira funtsezko lipidoek melanoman duten garrantzia aztertzeko, hala nola, PI fosforilatuak, glukosfingolipidoak eta esterolak.

Taula 8. Konparazio estatistikoetan euren mailak esanguratsuki aldatuta dituzten lipido espezieen zerrenda. *-ren koloreak espezie bakoitza zein azterketa-taldean den ugariago adierazten du. Zelula osasuntsuak (larruazal eta nevus melanozitoak): more; zelula gaiztoak (melanoma primario eta metastatikoak): marroi argia; larruazal melanozitoak: verde; nevus melanozitoak: hori; melanoma primarioak: gorri; melanoma metastatikoak: urdin. Esangarritasuna: *<0.05; **<0.01; ***<0.001; ****<0.0001.

Lipido azpimota	Lipido espezie	Osasuntsu vs Gaizto	M vs N	M vs MP	N vs MP	M vs MM	N vs MM	MP vs MM
SM	Lipido 1	***		**	***	**	***	
	Lipido 2				*			
	Lipido 3	***		*	**		*	
	Lipido 4	**	*	***	*	***		
	Lipido 5	**		*	***	*	***	
	Lipido 6	****		*	**	*		
	Lipido 7	***		****	****	***	****	
	Lipido 8	***			**		*	
	Lipido 9				*			
	Lipido 10	****						*
	Lipido 11	***						*
	Lipido 12	**			**		***	
	Lipido 13	***		*	****		****	
	Lipido 14			*	*		*	
	Lipido 15	****			***		**	
	Lipido 16	**			****		***	
	Lipido 17	***			****		****	
HexCer	Lipido 18					*		
DG	Lipido 19	****				**	**	
	Lipido 20	***			*	***	****	
TG	Lipido 21						*	
	Lipido 22							*
PC	Lipido 23	***				*		
	Lipido 24			*		*		
	Lipido 25	***		*	*	*	****	
	Lipido 26	***		****	***	***	***	
	Lipido 27	***				**	*	
	Lipido 28		**	***		****		
	Lipido 29	**					***	
	Lipido 30					**		
	Lipido 31	***		**	**	***	**	
	Lipido 32	**						
	Lipido 33	***			**		**	
	Lipido 34	**					*	
	Lipido 35	**						
	Lipido 36	*		**		**		
	Lipido 37	**			*		**	

	Lipido 38	***			****		****	
	Lipido 39	****				***	**	
	Lipido 40	***				*		
	Lipido 41					*	**	
	Lipido 42	***						
	Lipido 43						*	
	Lipido 44	***		**	**	***	****	
	Lipido 45	****			**		**	
	Lipido 46	****						
	Lipido 47	***		*		**	**	
	Lipido 48	***		*		**		
	Lipido 49						*	
	Lipido 50	****						
	Lipido 51	****		***		**		
	Lipido 52	****						
	Lipido 53		**	**		***		
	Lipido 54		**	**		**		
	Lipido 55		***	***		**		
PC eter	Lipido 56	****						
	Lipido 57		*			*		
	Lipido 58	****		*	*	*	*	
	Lipido 59	****		*	*	**	**	
	Lipido 60	***		**	***	**	****	
	Lipido 61	***				**	**	
	Lipido 62	**		*		***	***	
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	Lipido 64					*		
	Lipido 65	***						
	Lipido 66	***		**	**	***	***	
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	Lipido 68	***				*	*	
	Lipido 69	***						
	Lipido 70	****		*		*		
	Lipido 71	***		*		*		
	Lipido 72	***	*			*		
	Lipido 73					*	*	
	Lipido 74	***				*	*	
	Lipido 75	***				*	*	
	Lipido 76	***				*	*	
PE	Lipido 77					*		
	Lipido 78	***		*		**		
	Lipido 79	***						
	Lipido 80	**		**	**	**	**	
	Lipido 81	***	*	*		****		

	Lipido 82			*		**		
	Lipido 83	***		*	*	**	**	
	Lipido 84	***			*			
	Lipido 85	***		**	*	*	*	
PE eter	Lipido 86	**	*			**	*	
	Lipido 87		*					
	Lipido 88	**			****	*	****	
	Lipido 89	**						
	Lipido 90	****					**	
	Lipido 91	***			**			
	Lipido 92				**			
PC/PE	Lipido 93	****						
	Lipido 94	****					*	
	Lipido 95					***		
	Lipido 96	***		**	*	**	*	
	Lipido 97					*		
	Lipido 98	****				**	*	
	Lipido 99	****				**	***	
	Lipido 100	**		*	*	***	***	
	Lipido 101	****						
	Lipido 102	****						
	Lipido 103	****				***	**	
PC/PE eter	Lipido 104	***					*	
	Lipido 105	***					**	
	Lipido 106	****						
	Lipido 107	***		**	**	**	***	
	Lipido 108	****						
	Lipido 109	****		*	*	*	*	
	Lipido 110	***				**	**	
	Lipido 111			**		**		
	Lipido 112	****						
	Lipido 113	***						
	Lipido 114	****			***		**	
PS	Lipido 115					***		
	Lipido 116	***						
	Lipido 117	***		**	**	*	**	
	Lipido 118	****		**		****	*	
	Lipido 119	****				**	*	
PG	Lipido 120	**	*	**	*	***	**	
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	Lipido 123	***			*		*	
	Lipido 124	****			*		*	
	Lipido 125					***		

	Lipido 126	***						
	Lipido 127	***				**	*	
PI	Lipido 128	***						
	Lipido 129	**						
	Lipido 130	***		*	*	**	**	
	Lipido 131	***		*	*	**	**	
	Lipido 132	****		*	*	*	*	
	Lipido 133	***		**	**	**	*	
	Lipido 134	***			*		****	
	Lipido 135	**			**		*	
	Lipido 136	****		*	*	****	***	
	Lipido 137	***				**	**	
	Lipido 138	****				*	**	
	Lipido 139	***		**	**	****	**	
	Lipido 140	***				****	****	
	Lipido 141	**				****	****	
	Lipido 142	****			*	*	**	
	Lipido 143	****						

3. D2 fosfolipasak melanomaren ezaugarri protumoral eta prometastatikoak sustatzen ditu

Fosfolipidoek zelula-mintzetan burutzen duten egiturazko paperaz gain, metabolizatu eta bigarren mezulari ere bihur daitezke. Fosfolipasak fosfolipidoen ester edo fosfodiester loturak hidrolizatzen dituzten entzimak dira. Entzima horiek egitura-funtzioan eragiten dute bigeruzen sintesian, organuluen degradazioan eta biogenesian parte hartuz. Horrez gain, molekula bioaktiboen ekoizpenean ere parte hartzen dute[182]. PC, PE, PI, PG eta PS-en erreakzio katabolikoak A, C eta D fosfolipasek arautzen dituzte. Fosfolipasa bakoitzak, nagusiki, GPL mota bat hidrolizatzen du eta seinaleztapen-bide espezifikoak erregulatzen ditu, baina guztiek zelulen zorian parte hartzen dute, hainbat funtzio arautzen baitituzte, hala nola, hazkuntza, biziraupena, migrazioa, besikula-trafikoa, hantura, gaiztotzea eta metastasia. Izan ere, entzima horien adierazpena eta jarduera handitua aurkitu dira minbizi ugaritan. Beraz, ikertutako melanoma zelula lerroetan GPL azpimota ezberdinen maila aldatuak, fosfolipasek melanoman jarduera eta adierazpena aldatuta izan zezaketenaren hipotesia proposatu zen, GPL horiek fosfolipasen substratuak baitira. Western blot bidez PLA2, PLC, PLD1 eta PLD2 adierazpen-mailen azterketa egin zen gure zelula lerroetan. Emaitzetan PLD entzimek, bereziki PLD2 entzimak, larruazaleko melanozitoetatik melanoma metastatikorako trantsizioan mailak handitzen dituela frogatu zen. Gainera, melanoma zeluletan PLD2-ren gainadierazpena immunofluoreszentzia bidez berretsi zen. Gomez-Cambronero et al-ek minbizi ehunetan PLD entzimen adierazpena ez ezik, jarduera entzimatikoa ere handitzen dela adierazi zuen[138]. Ezaugarri hori gure melanoma zeluletan ere egiaztatu zen, lerro zelular primario eta metastatikoetan PLD jarduera nabarmen handitzen dela frogatu baikenuen.

PLD entzimek, zelula-mintzetan dauden PC molekulak hidrolizatu eta seinale mitogeniko eta biziraupeneko seinale bihurtzen dituzte. Konkretuki, PLD-ak tumore zelulen migrazioa eta metastasi handiagoarekin lotu dira; izan ere, minbizi-zelulen progresioan, zitoeskeletoaren dinamikan, mintzaren birmoldaketan eta zelula-ugalketan parte hartzen baitute, besteak beste[137]. PLD bidez PC-a apurtu ondoren, kolina bat eta PA molekula bat askatzen dira. Lehen aipatu bezala, PC-ak nabarmenki gainadierazita aurkitu dira aztertutako melanoma zeluletan, eta PLD bidezko kolina askearen ekoizpena PC molekula gehiago sortzeko erabil liteke, prozesu tumorala hobeto sustatzen duten azilo-kate zehatzekin. Gainera, prozesuan sortutako PA molekulak bigarren mezulari lipidikoak dira. Jakina da PA-k proteina ezberdinekin elkarrekintzan diharduela, seinale mitogenikoak sortzen dituzten Akt eta mTOR-rekin kasu[133,183]. Bestal, PA desfosforilatu ere egin daiteke DAG sortuz edo hidrolizatu daiteke Liso-PA sortuz; biak seinaleztapen-lipido indartsuak dira eta gaiztotze prozesua errazten dute[184]. Izan ere, PA maila altuak aurkitu genituen lehen deskribatutako ikuspegi lipidomikoetan. Hala ere, lipidoen azpimota hau ez zen analisi estatistikoan sartu, ezin izan baitzen bere jatorria baieztatu, lipido aitzindari baten zatiketaren ondoren detektatu ahal izan baitzen[185].

PLD entzimek PIP_2 kofaktorea behar dute beren jarduerarako, eta PI molekulek melanoma zeluletan presentzia handiagoa dutela frogatu dugu, ziurrenik PI-fosforilatuen mailak handitzen lagunduz. Izan ere, Epand et al-ek PI zikloak PLD jarduera handitzen duela frogatu zuten[61]. Gure aurreko emaitzen eta PLD jarduera handitzearen arteko beste lotura bat Diaz et al-ek baieztatu zuena da: esfingomielinasek nabarmen handitzen dute PLD-ren jarduera[186]. Gure emaitzetan ikusitako SM/Cer erlazioa murrizketa melanoma zeluletan, SMasa-ek melanoma zeluletan aktibitate handiagoa izan dezaketela iradokitzen du; beraz, ezaugarri horrek zelula gaiztoetan PLD jarduera handiagoa izatea ere lagundu lezakeela proposatzen dugu. Gainera, esfingosina-1-fosfatoa (S1P) ere PLD aktibatzaile gisa proposatu da[136]. Gure UHPLC-ESI-MS/MS emaitzek melanoma zeluletan zeramida maila baxuak daudela adierazten dute, neurri batean zeramidak S1P bezalako molekula pro-tumorigenikoetan bihurtzearen ondorioz azal daitekeena.

PLD1 eta PLD2-ren adierazpena eta jarduera altuak minbizi mota ezberdinetan deskribatu da, melanoma barne[139]. Egile batzuek PLD1-ek mikroinguru tumoralean eragin handiagoa duela iradokitzen dute; PLD2-k, berriz, tumore-zelulei eragiten diela batez ere melanoman, bularreko eta biriketako minbizian, besteak beste[145]. Beraz, Western blot, immunofluoreszentzia eta entzima-jardueran lortutako emaitzetan oinarrituta, PLD2 entzima melanoma primario eta metastatiko zeluletan gainadierazi eta isilarazi genuen, entzima horrek oinarrizko tumore-prozesuetan (zelulen hazkuntza, migrazioa eta inbasioa) duen eragina aztertzeko. Gure emaitzek, PLD2 jarduera eta adierazpena handitzeak hazkuntza, migrazioa eta inbasioa nabarmen areagotzen dituela erakusten dute, PLD2-ren isilpenak prozesu horiek murrizten dituen bitartean. Emaitza horiek bat datoz beste egile batzuen ekarpenekin. Linfoman[146] eta bularreko minbizi zeluletan[139], PLD2-k zelulen fenotipo ugalkorra eta inbaditzailea sustatzen dituela frogatu da. Izan ere, PLD2-k prozesu metastatikoa sustatzen du FAK fosforilatuz eta mTOR eta Akt aktibatuz[146]. Garrantzitsua da nabarmentzea PLD jardueran eta analisi funtzionalen artean hautemandako aldakortasunaren arrazoia, lipasa jarduera PLD2-ren ekintza-mekanismo bakarra ez dela izan litekeela. Izan ere, PLD1 ez bezala, GEF jarduera PLD2-ren ezaugarri da, eta hori oso garrantzitsua da zelulen mugigarritasunerako, besteak beste[135,137]. PLD2-k minbizian duen eragina horrela laburbil daiteke: biziraupen-seinaleak areagotu, hazkuntza handitu, MAPK, Akt eta mTOR-en seinaleztapen bideak aktibatu, apoptosiaren aurkako erresistentzia, angiogenesia, tumoreen hazkuntza zailtzen duten faktoreak ekidin, zelulen inbasioa eta metastasia sustatu, eta energia zelular etendua[137].

ONDORIOAK

1. Nevus eta azaleko melanozitoek eduki lipidiko global bereizgarria dute melanoma primarioko eta metastatikoko zelulekin alderatuta, eta horrek melanomaren garapenean zehar metabolismo lipidikoaren egokitzapen berri bat dagoela iradokitzen du.
2. Antzemandako aldaketa lipidiko garrantzitsuenak, melanoma primario eta metastatikoetan esfingomielina, triglizerido eta gantz azido aske espezieen gutxitzearekin lotuta daude. PI espezieen presentzia, ostera, areagotuta dago melanoman, bereziki lerro zelular metastatikoetan.
3. Mikroarraien teknologia erabiliz, zelula normalen eta tumoralen konparazioan mailak esanguratsuki ezberdinak dituzten 116 lipido espezie detektatu ziren, eta horiek melanoma-biomarkatzaileen hautagai potentzialak dira.
4. Mikroarraiak erabilita, hiru lipido espezie identifikatu ditugu, melanoma metastatikoetan horien mailak nabarmen handituta dituztenak melanoma primarioekin alderatuta; horiek pronostikorako biomarkatzaile-hautagai potentzialak dira.
5. Mintz funtzionalez osatutako mikroarraien teknologia berria azterketa lipidomikoetarako tresna bioteknologiko translazional egokia da, eta bere erabilera beste ehun eta patologia batzuk aztertzeko zabaldu daiteke.
6. PLD2 entzimak adierazpen eta jarduera handiagoa du melanoma zeluletan, bereziki melanoma metastatikoetan. PLD2-k zelula horien hazkuntza, migrazio eta inbasio ahalmena sustatzen ditu, zelula horien gaiztotze prozesua faboretuz.

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