DISEASE BIOMARKERS IN METABOLOMICS

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Castellón de la Plana, July 2020

Summary

Metabolomics aims to study the biological processes that take place in human body and their outcome molecules, the metabolites. Nowadays, the importance of metabolomics is in an upward trend because it has a huge number of clinical and analytical applications. Along this work, several topics will be treated. From the different biological matrices where metabolomics can be applied to some of the main known disease biomarkers. To finish specific characteristics of diseases such as ovarian or breast cancer will be discussed.

Keywords

Metabolomics, metabolites, biological processes, ovarian cancer

Index

1- Introduction

1.1- Justification

The main reason to choose this project is its modernity as well as my personal interest about this hot topic. In the last years, many studies have been performed showing that metabolomics is a very useful tool in the diagnostic of several diseases. Furthermore, applications in prognostic have also been found. These scientific advances are important for all of us because they could be the main tool for discovering new diagnostic and prognostic disease biomarkers in the next years.

1.2- Objective

The main objective of this work is to research and write a review about metabolomics and their applications. Besides, it also deeps intoens some aspects such as the different biological matrices analysed or specific disease biomarkers discovered.

1.3- Methodology

To carry out this work I have searched in different academic sources such as books and journals in order to be able to explain what metabolomics are, as well as their characteristics and most relevant aspects.

Despite having some general ideas about metabolomics, I have had to deepen my

knowledge in this interesting topic. I have also used websites and academic articles that have been of great help in understanding the whole metabolomics workflow.

1.4- Acknowledgements

First of all, I would like to appreciate the help and the advices that my supervisor Juan Vicente Sancho Llopis has given to me. I also want to mention all my degree teachers and express thanks to them for everything that i have learned about chemistry.

2- Introduction to metabolomics

2.1- Definition

Metabolomics are a branch of the called “omics cascade” which aims to study and research about metabolites in biological samples and how that could help to know more or to prevent diseases. Those metabolites are many different small molecules that take part in general metabolic reactions and in the normal function of every cell.
The “omics cascade” comprehend genomics, transcriptomics, proteomics ,
and metabolomics, from more to less complex analytes. These sciences will guide ourselves to a better comprehension of the biochemical and biological mechanisms that happen in living organisms. Although the metabolome involves a big complexity in its study as a wide variety of biomolecules and biochemical mechanisms are

implicated, it is a fact that in the last years the number of metabolomics publications has increased dramatically. Therefore, metabolomics has reached its maturity and it is achieving more importance every day.

Nowadays, there are two main approaches: metabolic profiling and metabolic fingerprinting.

2.2- Metabolic profiling and metabolic fingerprinting

The metabolic profiling approach studies directly a target group of metabolites that typically take part in the same metabolic pathway such as the metabolites present in the Krebs cycle, or those which come from fatty acids. The metabolic profiling [1] is a quantitative analysis that comprehend targeted analysis of a few number of analytes. This approach is often used in a driven-hypothesis rather than generating another one. Lately an effort has been made to build databases of these metabolites with the objective of improving the understanding about biological pathways, but it is a quest not completed yet.

In the other hand, we have the metabolic fingerprinting which is more a qualitative analysis (it allows to study simultaneously a wide range of metabolites). The aim of this approach is to identify patterns of analytes in the way to observe variations caused by an intoxication, a disease, or a change in the diet. A good example for the application of this approach could be the monitoring of a disease in humans. Observing the two metabolic patterns (one for healthy patients and another for ill patients) we could know, for example, if an specific treatment has been successful or not and prevent unexpected relapses.

It is remarkable, not only the metabolic fingerprinting of intracellular metabolites, but the metabolites that are excreted by the cells to the intercellular medium which could give big amounts of information about phenotypical changes. This approach is called

metabolic footprinting.

Combining both tools (profiling and fingerprinting) it is possible to find new biomarkers. Acquire biological knowledge and, at the same time, diagnose diseases and evaluate the pharmacological efficiency could be the results of a correct integration of both approaches .

2.3- Metabolomics workflow

From NMR to direct MS or injection into chromatography coupled to MS, a variety of instruments have been used for metabolomics applications. Mass spectrometry analytical platform has been proved to be the one which produce better results. Mostly comparing with NMR, MS analysis is able to study a wider range of biomarkers in the way of its higher selectivity and sensitivity. However, a sample preparation previous step is needed for MS-coupled analysis [1]. Typically, the acquisition in both LC-MS and GC-MS is performed with the aim to widen the chemical domain of the measured metabolites.

Actually, the obtained data is as valid as the sample preparation and experimental workflow and design.

Sampling is a key step in metabolomics workflow due to the influence of biological variability and the need of the obtaining of statistically validated data. In particular, when studying human samples, the influences of diet, gender, age and genetic factors have to be considered. In order to assure the representativity of the samples, quality controls samples such as samples replicates or blank samples must be analyzed. Also minimizing the formation or degradation of metabolites after sampling due to remaining enzymatic activity or oxidation processes are required.

Sample preparation is the next step in metabolomics workflow, mainly it will extract the analytes from complex biological matrices (plasma, serum, urine, saliva among

others) with the purpose of convert them into a compatible form with the analytical equipment. In the case of low-abundance metabolites, the extraction procedure can include a pre-concentration step to achieve the detection limits of the applied analytical technique [1]. Sample extraction should be as easy as possible in order to minimize the losses of analytes. It is important to say that in every sample extraction process analytes are lost, especially in extraction procedures applied to aqueous samples.Several methods could be used for this process and it only depends on the sample matrix. The main sample preparation processes are liquid-liquid extraction (LLE), solid-phase extraction (SPE), supercritical fluid extraction, accelerated solvent extraction, microwave-assisted extraction, protein precipitation, and membrane methods, such as dialysis or ultracentrifugation [1].

Once sample preparation is done, it is time to the data acquisition and its analysis. Both NMR and MS techniques follow two steps for data acquisition and processing. These steps are: 1) Processing of raw data with the purpose of peak identification, peak picking and normalization in order to have a comparison between samples. 2) Data analysis with the purpose of extract the analytical information and to elucidate biomarkers.

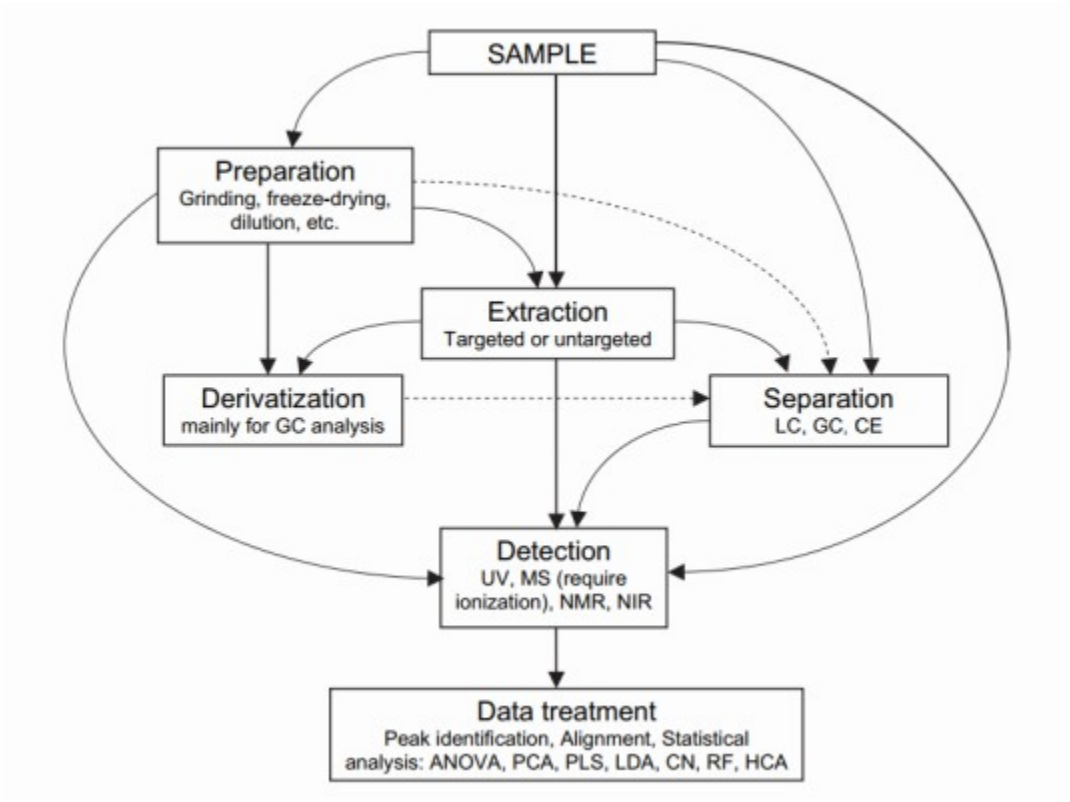


Figure 1: General metabolomic workflow

2.3.1- Instrumental equipment

MS-based techniques usually are the best option to carry out metabolomics and they have very different features [2].

1) High Performance Liquid Chromatography (HPLC) is the most used technique in the biological field. It is compatible with several stationary phases but reverse phase is the most used and it is able to retain hydrophobic molecules. However, samples in metabolomics could be biofluids which have many polar metabolites so reverse phase is not suitable on these circumstances. An option for the retaining of polar metabolites is hydrophilic interaction chromatography (HILIC), a new approach that is becoming popular because allows a complementary separation to the reverse phase.

Furthermore, in the last years the ultra-high-pressure liquid chromatography (UHPLC) has emerged. This separation method employs columns packed with particles of very small diameter (1.7μm) achieving a greater resolution for separation but increasing dramatically the pressure.

2) Electrospray ionization (ESI) is an interface which provides a gentle and sensitive ionization of the compounds. It is capable of ionize the molecules due to protonation and/or deprotonation processes

3) Quadrupole Time-of-Flight Mass Spectrometer (QTOF-MS) is a type of high resolution mass spectrometry (HRMS). Ions are blocked from the source and they are accelerated down to the detector. The ions which have a lower m/z would arrive first to the detector while higher m/z ions would arrive later. The time that an ion flies is correlated with its m/z with high mass accuracy. QTOF-MS systems consist of a quadrupole, a collision cell and a TOF analyzer, which allows to confirm known metabolites or elucidate some structures of unknown metabolites.

4) Ion Mobility Spectrometry (IMS) is a powerful technique that is usually coupled to a MS technique to achieve better results. The most remarkable coupling in metabolomics is the once with UHPLC quadrupole time-of-flight mass spectrometer (UHPLC-IMS-QTOF MS) system because it allows to maximize the detection of metabolites without increasing the analysis time. IMS is a gas-phase ion separation technique under atmospheric pressure based on their mobility which correlated with the 3D shape of the ion, adding an extra identification parameter, mainly for isomers discrimination.

However, NMR and MS techniques have advantages and disadvantages in metabolomic studies. While mass spectrometry has the advantage of being more sensitive than NMR (at the level of picograms), this sensitivity is not independent, because it is huge influenced by the pK of metabolite and its hydrophobicity. This feature might be a problem because while polar metabolites would be able to be detected with the action of the electrospray, the non polar metabolites may need atmospheric pressure chemical ionization. Moreover, some independent conditions

could affect dramatically the obtained results. These conditions use to be quenching, sample conservation and extraction.

In NMR analysis is preferable to avoid a complex sample preparation, especially in biofluids with low molecular weight biomarkers such as urine. Other biofluids which are plasma and serum need an extraction or a weight technique to divide the polar from the non polar metabolites. For MS analysis an exhaustive extraction is needed and many times the tissue is destroyed with the objective of eliminate all the interferences and picking up the wanted metabolites. Although it need a more invasive methodology, MS analysis usually reflects better sensitivity in metabolites detection.

2.3.2- Data processing

There are several data processing softwares which are able to process big amounts of raw data and they are used in many differents fields. They usually show 3D graphs, including retention time, m/z and intensity, but in metabolomics 4D graphs could be used with Progenesis QI software. The Progenesis QI program allows to include collision cross section in the shown data. The inner workflow for data processing in this software is: 1) Import data 2) Select of possible adducts present in your sample 3) Show graph of retention time vs m/z 4) Run alignment 5) Peak picking

2.3.3- Data analysis

Data analysis is the next and final step in metabolomic workflow. Although several software could be used for this step, they usually share principal component analysis (PCA) as main tool. PCA is a non-supervised analysis method with the aim of achieve a clear differentiation between samples with the least number of principal components and with the greatest degree of confidence. This method works identifying patterns, similarities and differences without any previous information about the samples content.

After PCA, a supervised analysis should be done with the purpose of improve the understanding of which variables are the responsible of the differentiation between groups. For supervised analysis previous information of sample is given. These supervised techniques are known as discriminant analysis and among them we can find the Partial Least Squares-Discriminant Analysis (PLS-DA) and orthogonal-PLS-DA (OPLS-DA). In PLS-DA method, the previous given information is used to distinguish between groups which have been already predetermined.

OPLS-DA method is used to create high predictive models in order to reprocess the data and facilitating the identification of potential biomarkers.

For biomarkers elucidation there usually are metabolite libraries available, which contain mass spectra of metabolites found in specific biological samples and found by a specific equipment such as GC-MS or LC-MS. In elucidation of new metabolites, additional analytical platforms will be needed. In the case of low-abundance metabolites, an alternative structure elucidation tool usually lacks the needed sensitivity. However, for high-abundance metabolites, proton or other types of NMR is the technique of choice for structure elucidation.

Figure 2: Data processing and analysis process

2.4- Biological matrices

Though metabolomics could be useful in almost every biological tissue or matrix, there are some typical biofluids, such as urine, serum, plasma and saliva or even breath.

2.4.1- Urine

Urine is a transparent yellow-coloured fluid produced by mammals kidneys. Kidneys filter bloodstream to take all kind of residues and excess water. The resulting urine has large concentrations of urea, salts, organic acids, ammonia and water-soluble toxins. Among all biological fluids, [3] urine is such an interesting one. It is because of its wide range of metabolites and due to the availability of large quantities of this fluid. As a biological waste fluid, it contains breakdown metabolites from foods, drinks, drugs, environmental pollutants and bacterial by-products. Besides, it is usually free from interfering proteins and lipids. However, there could be several variations between two different urine samples. It depends on more than 14 factors where diet, individual health and hydratation have been proved to be the most relevant.

For example, the urinary excretion of trans-ferulic acid (a polyphenolic derivative) increases after the ingestion of breakfast cereals and chocolate.

Although urine concentration variability makes data normalization a must, [4] at least a half of the published articles between 2012 and 2014 did not include any information about normalization.

Strategies were developed with the aim of estimate the urine concentration: (i) relative concentration to creatinine,. (ii) measurement of the total solute concentration and. (iii) urine/pure water density ratio. These strategies work better when they are combined.

In addition to the urine concentration variability, analytical drift for MS coupled methods has also been observed. To compensate this effect, QCs (quality controls) are used to assure the data stability during the experiment. These QCs are prepared mixing all the samples analysed in the same batch, therefore its composition represents the average of all the samples.

Nowadays, more than 3000 different metabolites have been identified in human urine and the number is still increasing.

Gagnebin *et al.* [4] reported the more abundant metabolites in urine. Theise values were normalized as relative concentration to creatinine (10.4 ± 2.0 mM). They are urea (22.5 ± 4.4 mM/mM creatinine), hippuric acid (298.5 ± 276.8 mM/mM creatinine) and citric acid (280.6 ± 115.2 mM/mM creatinine).

Observing some of the less concentrated metabolites (around 1 pM/mM creatinine) we found that current lower limit of detection in UHPLC-MS for metabolites in urine is in the low pM/mM creatinine range. This paper also assure that NMR may currently be the most comprehensive and certainly the most quantitative approach to characterizing this biofluid. However, NMR technique has also drawbacks such as the low sensitivity.

Chemical superclass	# Compounds
Aliphatic Acyclic Compounds	93
Aliphatic Heteromonocyclic Compounds	43
Aliphatic Heteropolycyclic Compounds	40
Aliphatic Homomonocyclic Compounds	18
Aliphatic Homopolycyclic Compounds	5
Alkaloids and Derivatives	45
Amino Acids, Peptides, and Analogues	286
Aromatic Heteromonocyclic Compounds	67
Aromatic Heteropolycyclic Compounds	728
Aromatic Homomonocyclic Compounds	432
Aromatic Homopolycyclic Compounds	6
Carbohydrates and Carbohydrate Conjugates	116
Homogeneous Metal Compounds	45
Homogeneous Non-metal Compounds	15
Inorganic compounds	1
Lignans and Norlignans	12
Lipids	866
Mixed Metal/Non-metal Compounds	7
Nucleosides, Nucleotides, and Analogues	49
Organic Acids and Derivatives	108
Organic Halides	3
Organometallic Compounds	1
Organophosphorus Compounds	17
Polyketides	74
Tannins	2

Table 1: Classification of determined metabolites in urine

2.4.2- Plasma

Plasma is the non-cellular part of blood. It is the result of removing all cells and only the non-living parts of blood remain. Its composition varies between different living species biut also between people. In general, it is formed mainly by water (around 90%) and biomolecules such as proteins, lipids, vitamins or glucose among others.

A study [5] was developed to determine the number and relative concentration of different plasma metabolites. LC/GC-MS technologies were used for that determination in 269 healthy subjects with different ancestries. From Caucasian to

Hispanic and Afro-American males and females aged 20-65. Ethylenediaminetetraacetic acid (EDTA) plasma samples were collected in New York. The results dropped that over 300 metabolites were identified and confirmed by using LC (60%) and GC (40%). Furthermore, they show that at least 100 compounds suffer a huge variability with age, sex or race.

Concentration increase in citric cycle intermediates (isocitrate, D-ketoglutarate and malate) for aged subjects. Significant differences in several cofactors and vitamins were observed as D-tocopherol, pantothenate and nicotinamide were higher in older subjects too.

Metabolites changes between sex were found to be very few. Around 30 metabolites present significant concentration difference between men and women. Ortophosphate, creatine and biomolecules associated with lipid metabolism such as nonanedioate, myristate, palmitoleate, glycerol and E-hydroxybutyrate were in higher levels in women. Generally, amino acid metabolism, energy metabolism and nucleotide metabolism were elevated in males relative to females.

Another important variable to study was the body mass index (BMI). A bigger change in some of those "variable" biomarkers was found into subjects with higher BMI than depending on sex, race or age.

Nowadays techniques have been developed increasing the qualitative detection range. By observing changes in the activation of specific enzymes, analytical instruments are able to find and identify more than 3000 plasma metabolites. In 2012 [6], an experiment based on the inactivation of xanthine oxidoreductase (XOR) (an enzyme that catalyzes the final steps in purine degradation) was carried out. The experiment was performed with LC-MS coupled with a multivariate statistical data analysis platform. In order to assure de reproducibility of the experiment 56 analyses were performed. The results confirm that XOR is not only the terminal enzyme in purine metabolism, catalyzing the oxidation of purine metabolites to uric acid, but it has also been implicated as a determinant of adipogenesis and other key metabolic processes.

2.4.3- Serum

Plasma is obtained from a blood sample, if anti-coagulants are introduced, by simply processes such as centrifuging the sample and removing or decanting the non-cellular portion. If no anticoagulant is added and the blood is allowed to clot, the supernatant fluid is called the serum [7]. Both, plasma and serum are mainly formed by water (around 95 %), but serum is less viscous because of his lack of coagulant proteins such as fibrinogen and prothrombin. Although they look very similar, the presence of cloting proteins changes hugely the identification of biomarkers. Psychogios *et al.* [7] suggests that most abundant metabolites in serum are D-glucose (5 mM), cholesterol (5 mM), melanin (5 mM), urea (4 mM), ATP (3 mM) and glyceraldehyde (1.5 mM). However, the values were into a huge range of variation probably caused by age, sex or others environmental factors. So, it is difficult to correlate one of those metabolites with a specific disease

Experimentally, a combination of different kind of methodologies was used to determine and identify the serum metabolites. Someo of those analytical platforms were high-resolution NMR spectroscopy, GC-MS, TLC/GC-MS, LC/MS, UPLC-MS/MS, and direct flow injection (DFI) MS/MS.

Psychogios et al [7] reviewed some of the analytical platforms results.

Regarding NMR experiments, they are simple and relatively easy to perform. Typically, around a 95 % of found metabolites could be identified.

For example, 53 serum samples were taken in an experiment by *Psychogios et al* [7]. The results showed that therey were identified an average of 30 biomarkers per sample.

For the GC-MS experiments, many of the peaks that appear into chromatograms were easily identified as well. However, around a 40 % of the peaks remain not identified. This relatively low level of coverage is a common problem in global or untargeted GC-MS caused by the lack of GC/MS metabolomics library. This lack is still a problem for other popular methodologies such as all kind LC-MS analysis.

Acyl glycines	10	Indoles and indole derivatives	12
Acyl phosphates	10	Inorganic ions and gases	20
Alcohol phosphates	2	Keto acids	8
Alcohols and polyols	40	Ketones	6
Aldehydes	3	Leukotrienes	8
Alkanes and alkenes	10	Lipoamides and derivatives	0
Amino acid phosphates	1	Minerals and elements	40
Amino acids	114	Miscellaneous	77
Amino alcohols	14	Nucleosides	24
Amino ketones	14	Nucleotides	24
Aromatic acids	22	Peptides	21
Bile acids	19	Phospholipids	2177
Biotin and derivatives	2	Polyamines	11
Carbohydrates	35	Polyphenols	22
Carnitines	22	Porphyrins	6
Catecholamines and derivatives	21	Prostanoids	23
Cobalamin derivatives	4	Pterins	14
Coenzyme A derivatives	1	Purines and purine derivatives	11
Cyclic amines	9	Pyridoxals and derivatives	7
Dicarboxylic acids	17	Pyrimidines and pyrimidine derivatives	2
Fatty acids	65	Quinones and derivatives	3
Glucuronides	8	Retinoids	11
Glycerolipids	1070	Sphingolipids	3
Glycolipids	15	Steroids and steroid derivatives	109
Hydroxy acids	129	Sugar phosphates	9
		Tricarboxylic acids	2

Table 2: Chemical classes in the Serum Metabolome

2.4.4- Saliva

Saliva is a biofluid which is mainly composed by water (99%) as well as. Several minor components including mucus, digestive enzymes, growth factors, cytokines, immunoglobulins, antibacterial peptides, bacterial cells and salts (Soini et al. 2010) [8] . However, its chemical composition varies dramatically in response of situational or environmental factors. Although saliva is very easy to obtain and it is abundant (on average, a person generates between 0.75 and 1.5 L of saliva per day), rarely is used for medical testing. The main reason is because it is not a not well known biofluid and there is a lack of chemical information about it. *Dame et al.* [9] were able to identify around 800 saliva metabolites by using a multi analytical platform approach and literature mining.

Some experiments were carried out to find these metabolites [9]. Samples were obtained from 16 subjects (8 males and 8 females) between 22 and 42 years. The subjects must not eat, drink, smoke or use oral hygiene products 1 h before the sampling. Samples were collected following Sugimoto *et al.* [10] protocol. Saliva was collected by spitting into a 50 mL Falcon tube for 3 min. The average volume collected was 2.5 mL.

For NMR spectroscopy, deproteinization is needed because substances such as mucous, proteins and other particulates can affect the sanalysis quality. The results showed that the most abundant compounds in human saliva are acetic acid (6.8 mM), propionic acid (1.4 mM), urea (0.61 mM) and L-lactic acid (0.53 mM).

For GC/MS, organic acids, polar and non-polar compounds were analyzed separately. To analyze organic acids, the ketoacids have to be converted to methoxime derivatives and then extracted twice with ethyl acetate and diethyl ether, followed by derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [3]. To analyze the non-polar metabolites, saponification with 2 M sodium hydroxide is needed, followed by heating. Most abundant compounds were the same than NMR analysis. However, number of identified metabolites by GC/MS were higher than in NMR, based on its much higher sensitivity.

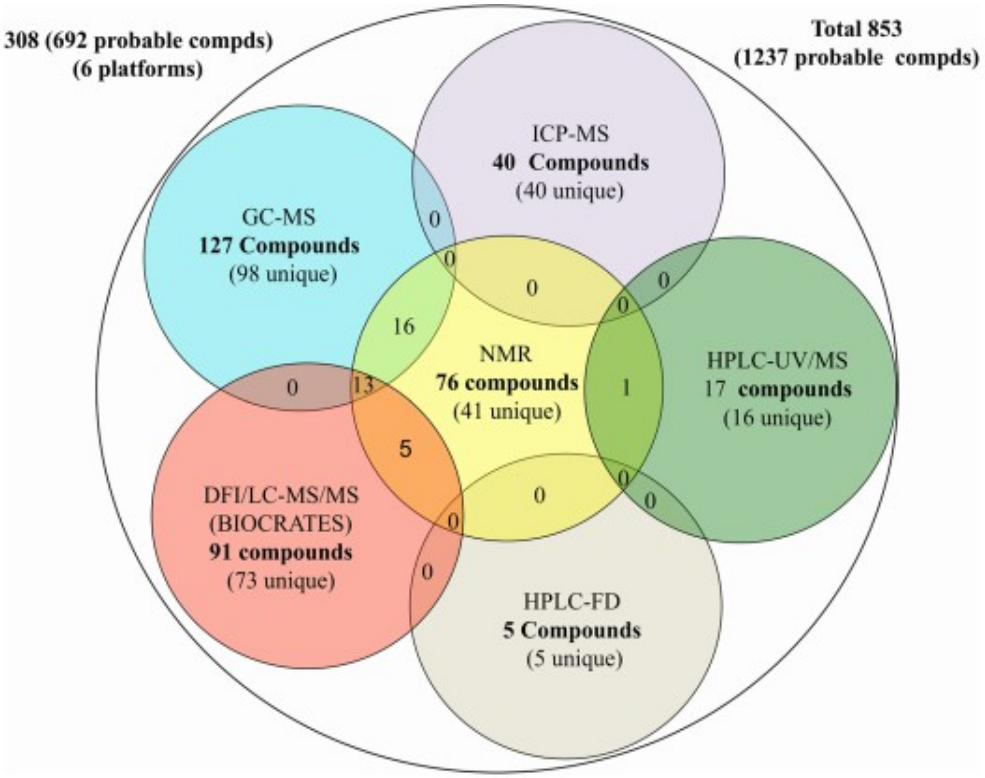


Figure 3: overlap of salivary metabolites detected by NMR, GC–MS, DFI-MS/MS, ICP-MS, and HPLC-coupled to MS, UV and fluorescence detection

2.4.5- Breath

Exhaled breath contains thousands of volatile organic compounds (VOCs) of which the composition varies depending health status, age, diet, and some other environmental factors. Those compounds have their origin into the blood when it reaches the lungs. Human brain is able to recognize more than 10000 different smells. Besides, it is able to recognize good and bad smells without knowing the chemical signals that is being registered.

Nowadays, modern technologies enable to identify some biomarkers present into the human breath.

Boots et al reported that one of the generally accepted exhaled biomarkers [11] is nitric oxide (NO), of which increased levels are associated with the occurrence of pulmonary inflammation and oxidative stress in various chronic lung diseases

including asthma and chronic pulmonary diseases (COPD).

Another researchable and waiting for further study biomarker is carbon monoxide (CO). Some studies, including *Yamaya et al*, found a correlation between CO exhaled levels and certain and important lung functions [12]. However, it is very difficult to assure because the measured CO concentrations are usually influenced by atmospheric CO.

Additionally, elevated levels of exhaled ethane and pentane are reported in patients suffering from several chronic lung diseases including COPD, asthma and cystic fibrosis.

Usually, the determination of only one biomarker has no phenotypical results, so the best way to understand the relevance of VOCs in human breath is to study all ofd those compounds. The total number of exhaled VOCs is also called as volatome. By studying the volatome we are able to comprehend processes underlying the pathophysiologies of interest.

In order to distinguish biomarkers of disease and environmental contaminant compounds, reliable analytical methodologies have been developed. Sampling is a sensitive stage, because it is easy to get interference compounds with the sample.

GC/MS is the preferred analytical technique because it is a commonly applied methodology to accurately measure trace gases in complex matrices. In addition, the GC-MS technique is proven to be highly sensitive and robust. Besides, it has huge degree of reproducibility.

Furthermore, use of GC-TOF/MS enables to deliver more spectral information because of the several advantages of using a TOF analyzer. Advantages of using TOF for broad spectrum analysis includes increased mass accuracy and mass resolution, greater sensitivity, rapid acquisition, and increased dynamic range when profiling over a broad molecular weight range.

Biomarker characteristic	Applying to VOCs
High sensitivity and specificity	++
Fast and accurate	+
Methodological simplicity	++
Interpretive simplicity	+/−
Thorough validation	++
(Patho)physiological link to either disease or exposure	+/−

Figure 4: Usefulness of VOCs as biomarkers on the basis of important biomarker characteristics.

2.5 – Present and future perspectives

Although some metabolomics applications have been already commented, a review of current and actual applications and future perspectives is needed.

Despite its utility in the clinical field, nowadays metabolomics is not still a must use technique in the health world. Metabolomics rarely outperform the traditional methods so it is difficult to integrate the needed technologies in the standard health services. However, their utility in the way of obtain a clearer sight of diseases (prognostic and diagnostic) and how they work [13], makes probable their integration in a close future. Furthermore, some associations in several countries are developing biobanks collecting metabolomic and other omics data with the purpose of carry out multiple layer analysis and assays. Those experiments would help to know more about biological processes and would give a clearer sight of several human diseases.

Recently, a set of metabolomic methods have been used for the creation of a disease risk model. This model rate the risk of suffer from a certain disease with a score. Its function is similar than the traditional model but with an outstanding accuracy. Currently, this model has been proved successfully for diseases, like pulmonary diseases, and in traits like educational attainment. In this methodology, simple assays are needed with the objective of follow up the disease development. For example, if a

patient has risk to develop blood cancer, periodical simple assays of carcinogenic cells would be carried out to follow up the disease. In addition, metabolomics is expected to increase its power and sensitivity and not only be used for diagnostic and prediction, but be a useful tool in disease treatment. For example, metabolomics could help the doctors to know if the patient is reacting well to an specific treatment or drug, or if a disease has probabilities to be reproduced again in the patient.

3 - Disease biomarkers

Advances in metabolomics allows to reach novel biological insights about metabolites and correlated phenotype. Following this way of knowledge, we are able to know more about disease biomarkers. Those biomarkers are very useful in clinical medicine for prognostic or predictive purposes. In the next sections, some biomarkers for stress oxidative diseases and for cancer disease are going to be commented.

3.1 - Oxidative stress related diseases

The oxidative or nitrosative stress is produced by the failure of the antioxidative cellular defenses to completely inactivate the reactive oxygen species (ROS) and the reactive nitrogen species (RNS). The failure could be caused by massive production of ROS/RNS, by a defect in the antioxidative defense system or both. This oxidative stress affects to a huge quantity of different biomolecules which are proteins, nucleic acid bases and lipids causing irreversible damages. These damages usually derive to affect cell health and also to the production of secondary reactives that drives to necrosis and apoptosis. Indeed, recent evidences have revealed that most of acute and chronical human diseases have stress oxidative origin.

Some studies [14] have shown that stress oxidative processes have a big influence

into aging process and aging diseases such as atherosclerosis, ophthalmologic and neurodegenerative diseases. Furthermore, it is known that aging is mainly caused by an accumulation of oxidized species in tissues. It seems to be a disparity in the concentration of those oxidized species between intracellular and extracellular medium. The extracellular medium shows a bigger level of those species because of the turnover between intracellular medium and because of the difference in homeostatic control. Biomarkers are defined as characteristics that can be measured and are representative of a biological process. ROS/RNS are not biomarkers because of their low stability and half-time life so the ones used as markers are the products of reaction between oxidative and nitrosative molecules and biomolecules. Some general biomarkers allow us to identify several diseases which have an oxidative/nitrosative origin.

3.1.1- MDA

MDA is a ketoaldehyde which is produced during the arachidonate metabolism as a byproduct of peroxidation decomposition of lipids. The accumulation of MDA in an injured tissue drives to a bond with some aminoacids (mainly with Lysine) altering proteins and their biological functions. MDA could be found in many diseases such as atherosclerosis or diabetes mellitus having a prediction function in the progression of those diseases also including coronary artery disease or myocardium infarct.

In atherosclerosis MDA interacts with the protein LDL to mediate several proinflammatory and proatherogenic processes that may generate foam cell leading to coronary heart disease and strokes.

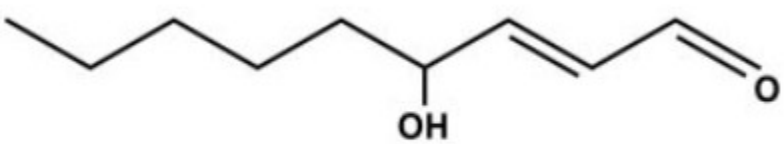
MDA

Figure 4: Molecular structure of MDA

Elevated concentrations of MDA adducts with Lysine have been found in samples of women patients with preeclampsia, and also in plasma and breath in patients with asthma. Besides, it has found huge levels of MDA in diabetes mellitus patients plasma and in brains of people who suffer from Parkinson disease. Related substances of MDA are the thiobarbituric acid-reactive substances which have been determined in high levels in human plasma of people who have amyotrophic lateral sclerosis or Alzheimer.

3.1.2- HNE

HNE is a toxic aldehyde produced by a free radical attack on omega-6-polyunsaturated fatty acids such as arachidonic or linoleic acids. HNE undergoes many reaction with biomolecules and it is cytotoxic, mutagenic and genotoxic. It also has several signal effects taking part in key processes such as inhibition of DNA replication and protein synthesis, or inactivation of enzymes.



HNE

Figure 5: Molecular structure of 4-HNE

The usual concentration of HNE in human body is in the range from 0.1 to 3 μmol/L, although in an oxidative stress situation its concentration raises hugely. HNE plays the role of biomarker in diseases like Alzheimer or atherosclerosis, together with MDA. It is not an exclusive aging-disease biomarker but it is also present in other illnesses such as mild cognitive impairment or rheumatologic diseases.

3.1.3- Isoprostanes

Isoprostanes are the result of a free radical reaction of peroxidation of arachidonic acid. F2-Isoprostanes are the most studieds due to their stability in human body. These molecules haves renal and pulmonary vasoconstrictor function and they are very useful as oxidative stress biomarkers in several health conditions. One of the most remarkable case where isoprostanes are good biomarkers is in rhabdomyolysis, a disease where myoglobin is released to plasma and then it can derive into a kidney vasoconstriction.

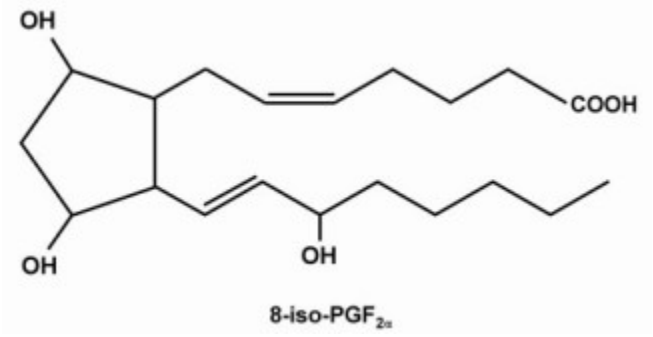


Figure 6: Molecular structure of 8-iso-$PGF_{2\alpha}$

Isoprostanes are easily found in almost every biological tissue or body fluids and they

are able to be determined with different instrumental equipments from MS techniques to immunoassays. The average concentration of each F2-Isoprostane in plasma are less than 10 ng/L and it is known that they work as biomarkers in diseases like cystic fibrosis, asthma, acute chest syndrome of sickle cell disease or acute and chronic alcoholic liver disease.

3.1.4- Glutathione

Glutathione and its derivate disulfide glutathione concentration are determined in blood analysis because it reflects the whole body status of these molecules. This determination is usually done by MS-based techniques but it could be also analyzed with other analytical techniques such as spectrophotometric and fluorometric assays. In contrast with the other mentioned oxidative stress markers, glutathione levels decrease when this stress happens, for example in aging. The decrease in glutathione concentration is also related with the pathogenesis of several diseases such as rheumatoid arthritis, amyotrophic lateral sclerosis, alcoholic liver disease, cataract genesis, respiratory distress syndrome or cardiovascular disease among others. Furthermore, high glutathione levels are related with a long life-span in some mammals and also in the elder population.

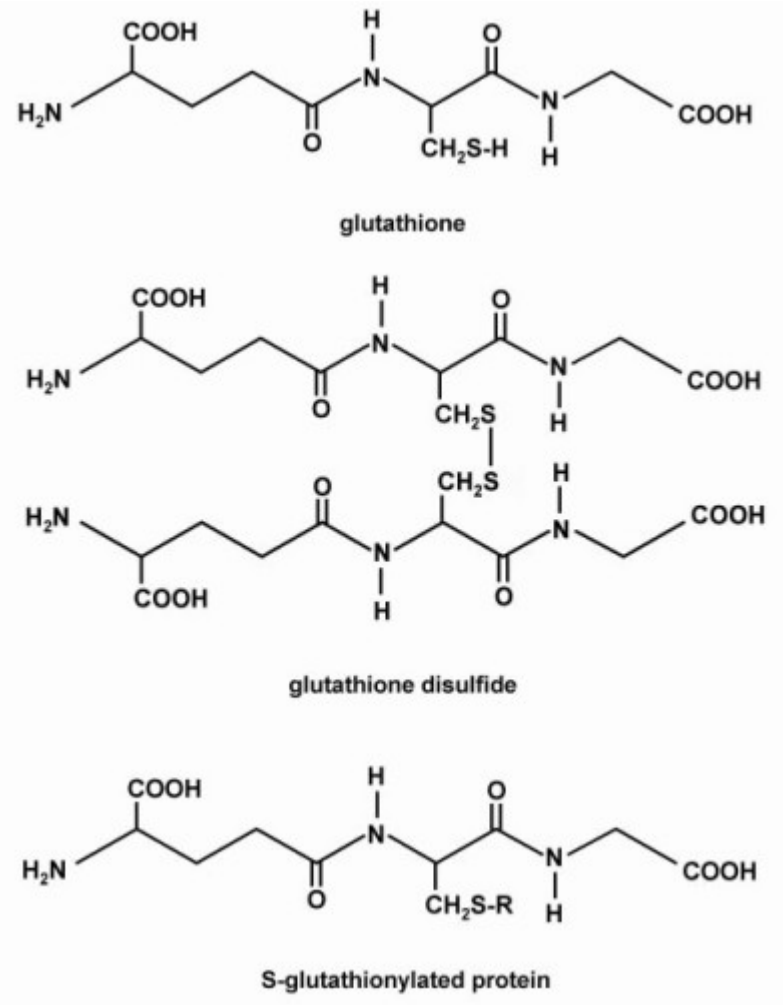


Figure 7: Molecular structure of glutathione and its derivatives

3.2- Cancer

Nowadays, several ways exist to do a metabolomic cancer analysis [15]. The scientists are able to carry it out *in vivo* and *in vitro* using a wide range of sample types from tissues to the above commented biological fluids. With the objective of reduce the difficulty of the analysis, biological fluids are usually used for their less complex matrice being urine and plasma the most used among them.

However, the interest of the direct metabolomic analysis of the malignant tissue is growing. Although, the complex task being the tissue preparation still remains the biggest problem. There could be many different interferences which might be caused

by the atmospheric conditions and the contamination from near healthy tissues.

It is important to know the common and general methodologies used in the metabolomic experiments or analysis. I is essential to maintain a constant temperature and to do the sample extraction taking care to avoid possible interferences. For biological fluids, the standard sample volume is in a range of 0,1-0,5 mL.

Remarkable discoveries have been done in the tumor metabolome in the last years. It is known that phospholipids levels (characterized by a raise in choline-containing compounds) and glycolytic functions are bigger than in a healthy tissue. Glycolytic functions include an increment into the consumption of glucose carbons to drive synthetic processes, high glutaminolytic function, and overexpression of the glycolytic isoenzyme, pyruvate kinase type M2-PK (probably a tumor biomarker). However, metabolome among different cancer types varies making it difficult to generalize findings across tumor groups. Furthermore, the characterization of a tumor could be hindered by several variations such as sample-to-sample variation or sensitivity in extraction-based MS methods. Some investigations [16] have shown that a malignant phenotype in breast cancer could be detected from normal tissue by choline-containing compounds determination with a sensitivity around 100 %. In addition, a 68 % of biopsies could have been prevented following the choline method. Another well-known case is prostate cancer, where the malignant tissue shows to have a reduced level of citrate and a raise of spermine. This case fits with the high concentration of choline substances pattern; moreover it has been detected high lactate and alanine levels (both are glycolytic products).

Despite its complexity, the knowledge about metabolomics in brain cancers is extensive. Depending on the tissue there several subtypes where meningiomas, neuroblastomas, and glioblastomas are the most commons. There are differences between these subtypes, mainly in concentrations of substances such as alanine, glutamate, creatine, phosphorylcholine, and threonine. Brain cancers presents also huge concentrations of total-choline compounds which could be used as a biomarker,

but other variation is noticed with the decrease of N-acetyl aspartate concentration.

Ovarian cancer is one of the diseases that is going to be comment later deeply. However, it is interesting to comment the results of a study [17] carried out with NMR spectroscopy and with samples from healthy women, women with benign ovarian cysts and women with preoperative ovarian cancer. The results showed that the profiling, both in serum and in plasma, was able to establish patterns that varied between the three sample groups. The next step was to determine if there was an evident variation between tissue invasive carcinomas and superficial tumors. The results dropped that at least 51 metabolites could be used as biomarkers for the identification of such an invasive malignant tumor from a borderline tumor. Furthermore, the differences noted in these metabolites have previously been linked to prognosis in ovarian cancer and correspond to pathways responsible for regulation of pyrimidine metabolism.

4- Ovarian cancer

In this work the metabolomics main facts and features and also the cases of some specific diseases such as oxidative stress diseases and cancer have already been commented. But, one objective about this work is to deep in to one specific disease which is ovarian cancer. Metabolomics bring the bright of hope to the health world because they make the scientists and doctors be able to predict a disease before it is too late or use as a tool to identify if a treatment has a good response in the organism, so it can be very useful to save lives.

Ovarian cancer (OC) is one of the leading causes of death from gynecologic diseases in the developed world. Around the 80 % of people who suffer the advanced form of this disease has a rate of survival from 15 % to 45 %. However, in patients who suffer this disease in his first stage exist a survival rate of a 90 %. This difference between stages mortality has motivated researchers to find new ways of diagnostic and

screening in order to identify the malignancy earlier to save lives. Although OC knowledge has been spread and increased, there is still not a screening method which ease the survival [18]. Some of the proposed methods are tests such as serum cancer antigen 125 (CA-125), ultrasound imaging, or combination of both of them. Nowadays, the screening with CA-125 is widely available but it has a low sensitivity and a low predictive value. The ultrasound imaging is not so specific with ana specificity range between 70 and 80 %, and it is also an operator-dependent screening so it has some issues. Despite a combination of both gives the best results, the technique is still under the required specificity and sensitivity levels of an effective screening technique.

Another important issue is the selected biological matrix for the metabolomic analysis. Despite several matrices give good results there are still some problems to solve. In the case of serum analysis, the metabolomic analysis of OC patients confirm that is able to improve the knowledge of the OC metabolome by identifying some new biomarkers with high accuracy. For urine samples, the metabolomic analysis shows the ability to identify some biomarkers of OC and distinguish them from breast cancer. However, with urine analysis by its own it is not possible to distinguish between OC and cervical cancer.

In order to elucidate the analyzed biomarkers and metabolites found in OC metabolome data libraries are used. Most popular databases are Human Metabolome Database, MassBank, KEGG and METLIN and, in addition, they are all free in Internet. The last step is to determine and quantify an specific biomarker depending on the researched aspect of the malignancy. This step is the most challenging of all, because the complexity of identify every metabolite that acts as biomarker [19]. However, in the last years several advances have been developed to make easier this key step. *Vermeersch et al.* affirmed that ability of metabolomics to measure high-throughput, system-wide phenotypes give it significant potential power in the field of oncology to understand cancer metabolism and carcinogenesis while allowing biomarker discovery-based analysis [20].

Several methodologies could be used for detect and quantify metabolic biomarkers. A targeted NMR approach was carried out by *Garcia et al.* to diagnose the levels of metabolites in patients serum with early OC [21]. There were 120 subjects and 132 controls. The results showed that there was an excellent separation between samples and controls. Moreover, a comparison with biomarkers of renal cell carcinoma was made and showed that this methodology was very sensitive to OC.

Another experiment [22] was performed using UHPLC-QTOF-MS to distinguish between biological patterns of early OC, benign tumors (BOT) and uterine fibroid (UT). With this methodology the scientists were able to discriminate OC from BOT with an area under the curve (AUC) of 0.910 and OC from UF with an AUC of 0.942. In addition, the profiling of metabolites in stage I showed that this method is able to distinguish from advanced stages of OC, being 53 metabolites which acted as biomarkers. The more significant are lysophosphatidylethanolamines (LPEs) and lysophosphatidylcholines (LPCs). In addition, the experiments showed that other metabolites have their concentration altered in early OC cases. Those biomarkers are imidazol-5-pyruvate, N4-acetylcytidine, pseudouridine, succinic acid, (S)-reticuline, N-acetylneuraminic acid, 3-sialyl-N-acetyllactos-amine, β- nicotinamide mononucleotide, and 3-sialyllactose. Besides, these biomarkers were not only useful to differentiate between early and advanced OC, but also with benign tumors and other ovarian diseases.

Several studies of the OC metabolomics revealed that there are also altered metabolites which proceed from different biological processes. These metabolites are mainly related with key biological pathways such as cellular respiration or carbohydrate metabolism, fatty acid oxidation, aminoacids metabolism and, nucleotide-related processes.

4.1- Cellular respiration metabolites

The cellular respiration metabolites are the single molecules that outcome from glycolysis, the Krebs cycle and anaerobic respiration. For these pathways, there are several changes in metabolites concentration. For example, the levels of lactate (metabolite derived from anaerobic glycolysis) increase in malignant cyst fluid, but seem to decrease in serum and urine.

Glucose levels undergoes changes too, they increase in serum while decrease in urine. However, glucose levels do not change in malignant cyst fluid and in malignant tissues neither.

For Krebs cycle (or TCA cycle) three metabolites seem to change their concentration in human body. Malate and fumarate levels usually increase in almost every biological fluid. In the other hand succinate shows contradictory results because it sometimes presents low levels and others an increasing is observed.

4.2- Fatty acid oxidation metabolites

Free fatty acids and the metabolites produced from their oxidation present huge changes in their concentration levels. Several studies have reported high levels in fatty acid oxidation metabolites, which are carnitine, acetylcarnitine, butyrylcarnitine, propionylcarnitine and other carnitine derivated metabolites.

In general, the group of cholines usually increase their concentrations in patients with early and advanced OC. Furthermore, this choline levels are bigger in early OC patients than in the advanced disease.

Other molecules such as ceramides, gangliosides and lysophosphatidic acid (LPA) were reported to be in high levels in serum, urine and ascitic fluid for early and advanced OC patients. Increased levels of long chain fatty acids and their esters have

been noticed in malignant tissues and serum. The more remarkable are palmitate, myristate and oleate.

To finish, *Buas et al.* [23] used lipidomics profiling and identified 17 metabolites including glycerolipids and glycerophospholipids that were decreased in OC cases.

4.3- Aminoacids metabolites

Amnoacids and aminoacids metabolism metabolites present changes in their concentrations, but not every metabolite presents a continuous variation. Some of these molecules are alanine and valine where some studies reported oscillation in their levels for OC patients.

Levels of aminoacids which are donors of nitrogen usually increase in serum, urine and metastatic tissue of people who have OC.

Zhou et al. [24] found increased levels of cysteine, glycine, and threonine using MS platforms.

Altered levels of metabolites and aminoacids involved in L-tryptophan metabolism was shown in OC with decreased plasma and serum levels of tryptophan and its metabolites 3-indolepropionic acid and 5-hydroxyindoleacetaldehyde as *Hilvo et al.* [25] reported. It was also found that in L-tryptophan degradations 3-indolelactic acid levels increased in urine and kynurenine levels increased in serum, plasma and malignant tissue.

Several metabolites with origin in phenylalanine catabolism present altered levels for OC early and advanced patients. Phenylpyruvate, phenyllactate, and phenylacetate are the mentioned metabolites. Phenylalanine levels in serum decrease because of their enhanced catabolism, but hippuric acid, a by-product of phenylalanine production, decrease its levels in urine.

The L-histidine, another aminoacid, appear to low its concentration in urine, serum

and plasma for early OC patients. Furthermore, the product of L-histidine metabolism, imidazol-5-yl-pyruvate, seems to be at higher levels in OC patients. This fact probably means that enhanced metabolism of this aminoacid occurs.
Finally, *Fong et al.* [26] confirmed that some brain aminoacids such as N-acetylaspartate (NAA) and its derivative N-acetylaspartatyl-glutatmate (NAAG) appear in higher conditions than standard levels for metastatic OC patients.

4.4- Nucleotide metabolites

The most of registered metabolomic patterns related with nucleotides and nucleotides metabolites are about regulation of purine and pyrimidine metabolism. *Chen et al.* [27] reported that pseudouridine was commonly found to be elevated in urine and serum, while hypoxanthine was found elevated in serum, malignant cyst fluid and malignant tissue. Elevated levels of 1-methyladenosine, 3-methyluridine, N4-acetylcytidine and urate-3-ribonucleoside in urine were also reported.

4.5- Other metabolites

In this section the more remarkable and abundant metabolites which are not related with an specific pathway are going to be commented.
First, several studies such as the carried out by *Hilvo et al.* [25] confirmed that ketone levels in serum usually increase in patients with OC. For example, 3-hydroxybutyrate levels seems to increase in high quantities specially for metastatic patients. Acetoacetate, acetone, 3,4- dihydroxybutyric and 2,4-dihydroxybutyric acid are other ketone bodies that show an increase on their levels and concentrations in human serum for people suffering from OC. In addition, other species such as 2-hydroxybutyric acid, and α, δ and γ-tocopherol have an elevation in their

concentration too.

Other studies have found altered histamine metabolites in early OC patients. Furthermore, *Zhou et al.* [24] demonstrated that a significant fraction from the metabolites found in serum of OC cases have their origin in histamine pathway.

4.6- Discussion

Main metabolites that act as biomarkers in key biological processes have already been commented. However, in this section this topic is going to be discussed in detail.

The OC cases present some altered metabolites for the cellular respiration in glycolysis, the Krebs cycle and anaerobic respiration and that reflects an alteration in energy obtaining. The alteration in energy metabolism was discovered almost a century ago and received the name of Warburg effect in honor of his finder. This phenomenon was demonstrated with several metabolomics analysis, confirming it with increased levels of glucose 6-phosphate and glucose 1-phosphate in cancer tissue. The Warburg effect is known to enhance the production of lactate and to reduce the pH of the cancer environment, causing impairs DNA repair mechanisms. However, in reviewed studies there is a lack in the consistence of lactate increasing and it is remarkable the increase of Krebs cycle intermediates (aerobic respiration) despite of Warburg effect. This phenomenon could be explained by anaplerosis process. As commented by *DeBerardinis et al.* [28] this mechanism refers to replenishment of intermediates through the generation of α- ketoglutarate from glutamate following its conversion from glutamine, generating macromolecules that are essential for cellular proliferation.

Moreover, the substrates of two enzymes which take part in Krebs cycle (succinate dehydrogenase and fumarate hydratase) are identified as onco-metabolites and the

inhibition of these enzymes leads to an increase of succinate and fumarate levels. The raise of these two metabolites has been clearly related with patients who suffer from early OC. These alterations of Krebs cycle intermediates and their enzymes suggest a big demand of energy, and that is a known feature about a malignant tissue which is growing.

Lipids metabolism is another key biological process that presents altered metabolites for OC patients. The main reason to relate ovarian malignancy with the altered oxidation of lipids is that the adipocytes act as a reservoir of energy for proliferant cancer cells. This fact was supported when beta oxidation of fatty acids appeared to be altered in several malignant tissues such as prostate and kidney [29]. Moreover, *Derdak et al.* [30] confirmed that increased fatty acid oxidation is associated with an over-expression of uncoupling proteins that can promote chemoresistance in cancer cells through mitochondrial "uncoupling," helping cancer cells to survive. This oxidation is manifested as an elevated concentration of carnitine proteins in serum, plasma and malignant tissue from OC cases.

The enhanced oxidation of lipids involves also an altered function of acetyl-CoA. This molecule usually enters in Krebs cycle, however due to the altered turnover of this process, metabolites leads to deplete oxaloacetate levels in the liver. This phenomenon drives in turn to an hindrance for acetyl-CoA to enter in Krebs cycle so the molecule is converted into ketone bodies which could be good biomarkers for early OC.

The enhanced fatty acid oxidation leads to altered metabolism of sphingolipid and glycerolipid as well as to altered levels of long chain fatty acids. Several studies [23] have confirmed by lipidomics profiling in OC samples that there is a decrease in at least 17 metabolites which are mainly glycerolipids and glycerophospholipids. Furthermore, elevated levels of lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA) were previously reported as possible biomarkers for early and advanced OC. In order to distinguish early OC from metastatic OC some studies carried out by *Ke et al.* [22] in 2015 revealed that there is a difference in LPCs and LPEs

(lysophosphatidylethanolamines) levels depending on how advanced is the disease. In early OC disease levels of LPCs and LPEs seem to be altered and they are higher than in metastatic OC showing a potential biomarker to distinguish between both health status and to know more about the progression of the disease.

The experiments of *Coussens and Werb* [31] confirmed that sphingolipids and fatty acids have cellular signal transduction activity that contributes to genetic instability and cancer initiation.

Amino acid metabolism is another key biological pathway that is commonly altered in cancer cells. For example, the high cell cycle turnover cause an increased uptake of glutamine in cancer cells. Glutamine is a key aminoacid because it plays a role as the amino group donor for many biosynthetic pathways. Not only glutamine but glutamate show altered levels in metabolic profiling studies of serum, cyst fluid, urine and tumor tissue. Other aminoacids such as alanine, glycine, threonine and cysteine show elevated concentrations in patients with OC. These aminoacids have a huge biological relevance because of their function in protein biosynthesis so their altered levels indicate the rapid cell cycling and high turnover of cancer metabolism.

The degradation of L-tryptophan in OC patients is one of the immune resistant mechanism that OC disease has. *Platten et al.* [32] said that the enhanced degradation of tryptophan leads to T cell anergy and apoptosis via the GCN2 pathway, while increased levels of the tryptophan breakdown product kynurenine suppresses T cell differentiation. This process has been studied largely during the years and it is confirmed by metabolomics studies (*Ke et al.* [22]) with decreased plasma levels of L-tryptophan and its metabolites (5-hydroxyindoleacetaldehyde and 3-Indolepropionic acid). Furthermore, principal metabolites coming from phenylalanine metabolism (phenylpyruvate and phenylacetate) seem to have elevated concentrations in the serum and malignant tissue of early OC while the levels of phenylalanine appear to decrease in serum (enhanced catabolism of phenylalanine). Additionally, benzoate degradation is known to be a part of phenylalanine pathway, which is associated with altered hippurate levels [22]. Decrease of hippurate levels in serum is

a common phenomenon in OC patients. Despite of the several OC-related findings in phenylanine pathway, there is still not clear the role in OC of this molecule and their metabolites.

Another substance, the L-histidine show decreased levels in serum and urine in profiling studies carried out by *Ke et al.* [22] and *Zhang et al.* [33]. Further, in these studies elevated levels of midazol-5-yl-pyruvate, a L-histidine degradation metabolite, which is probably caused by an enhanced metabolism of this aminoacid. In addition, the enzyme histidine decarboxylase converts histidine to histamine. Over the past decade, considerable data suggest an important function of histamine in malignant cell proliferation [34]. Histamine works as a receptor-dependent growth factor in malignant tissues for OC and also in breast cancer and in malignant melanoma.

Based on the nucleotide-related metabolites, it is a fact that nucleotide metabolism is enhanced in OC disease. The tumor growth is dependent from the synthesis of new purine and pyrimidine nucleotides. However, there is still not enough knowledge about nucleotide pathways in OC pathogenesis.

To finish, another altered metabolite in OC disease is the tocopherol. The isoforms α, β, δ, and γ of tocopherol are known to have antioxidant activity and possess a protective effect against many cancers [35]. *Fong et al.* [26] found increased levels of α ,δ and γ tocopherol in metastatic OC but he did not find altered concentrations of any tocopherol in early OC. This difference could be explained by the fact that this metabolic alteration develops during the metastatic stage.

5- Conclusion

In this work, the most important aspects of metabolomics have been discussed, from the most general ones to end up focusing on clinical metabolomics. Aspects such as the two types of metabolomic approaches with their advantages and disadvantages have been reviewed. The metabolomic workflow usually followed in this kind of studies has also been reviewed and the main analytical instruments used have been slightly commented. The main biological matrices that could be found in these analyses have also been treated. Afterwards, the current applications have been discussed along with a small perspective of how metabolomics could be useful for the medical world in the future. Finally, we have focused mainly on two groups of diseases, those caused by oxidative stress and cancers.In the last group of diseases we have gone even deeper since ovarian cancer was the target of the experimental work that could not be carried out due to the state of alarm declared in March.

It is important to add that our original work was mainly oriented towards the metabolomic approach of fingerprinting, as almost all references reviewed on OC are focused on profiling specific metabolites from presumable altered pathways.. The aim was to perform an external validation of a predictive model of optimal surgery in ovarian cancer. The aim was to perform an untargeted metabolomic analysis in order to optimize certain prognostic factors to predict the response to treatment. It was also intended to help improve and personalize treatments as well as to be able to relate the levels of certain metabolites as markers of recurrence when monitoring a patient after or during the treatment.

6- References

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