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Characterization of circulating steroids and endocannabinoids levels in obese subjects by liquid chromatography tandem mass spectrometry

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CHAPTER 3 – CIRCULATING ENDOCANNABINOIDS QUANTIFICATION IN OBESE PATIENTS BEFORE AND AFTER GASTRIC BYPASS SURGERY

ABBREVIATIONS

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11βHSD : 11β-hydroxysteroid dehydrogenase type 1

2AG : 2-arachidonyl glycerol

ACE : angiotensin converting enzyme

ACTH : adrenocorticotropic hormone

AEA : anandamide

AEXS : aromatase excess syndrome

amu : atomic mass unit

APCI : atmospheric pressure chemical ionization

API : atmospheric pressure ionization

AR : androgen receptor

AUC : area under the curve

BDNF : brain-derived neurotrophic factor

BED : binge eating disorders

BMI : body mass index

BSA : bovine serum albumin

CAD : collision activated dissociation

CAH : congenital adrenal hyperplasia

cAMP : cyclic adenosine monophosphate

CB1 : cannabinoid receptor type 1

CB2 : cannabinoid receptor type 2

CBG : corticosteroid-binding globulin

CE : collision energy

CNS : central nervous system

CRH : corticotropin-releasing hormone

CV : coefficient of variation

CXP : cell exit potential

DAG : 1,2-diacylglycerol

DAGL : diacylglycerol-lipase

DBP : diastolic blood pressure

DHEA : dehydroepiandrosterone

DHEA-S : DHEA-sulphate

DHT : dihydrotestosterone

DOC : deoxycorticosterone

DP : declustering potential

E_2 : estradiol

ECS : endocannabinoid system

ECs : endocannabinoids

EP : entrance potential

ER : estrogen receptors

ESI : electrospray ionization

EU : European Union

FAAH : fatty acid amide hydrolase

FAK : focal adhesion kinase

FDA : Food and Drug Administration

FLAT : FAAH like anandamide transporter

FSH : follicle-stimulating hormone

GC : gas chromatography

GC/MS : gas chromatography coupled with mass spectrometry

GI : gastro-intestinal tract

GLP-1 : glucagon-like peptide1

GnRH : gonadotropin-releasing hormone

GR : glucocorticoids receptors

HbA1C : glycated hemoglobin

HPA : hypothalamic-pituitary-adrenal axis

HPG : hypothalamic-pituitary-gonadal axis

HPLC : high pressure liquid chromatography

HRE : hormone responsive elements

IS : internal standard

ISTD : deuterated internal standards

LC : liquid chromatography

LC-MS/MS : liquid chromatography tandem mass spectrometry

LH : luteinizing hormone

LLE : liquid-liquid extraction

LLOQ : lower limit of quantification

LOD : limit of detection

m/z : mass-to-charge ratio

MAGL : monoacylglycerol lipase

MALDI : matrix-assisted laser desorption/ionization

MR : mineralocorticoids receptors

MRM : multiple reaction monitoring

mRNA : messenger RNA

NAEs : N-acylethanolamines

NAPE : N-arachidonoyl- phosphatidylethanolamine

NAPE-PLD : NAPE-phospholipase D

NAT : N-acyltransferase

NF-kB : nuclear factor kappa-light-chain-enhancer of activated B cells

NPY : neuropeptide Y

OEA : oleoyl-ethanolamine

PCOS : polycystic ovary syndrome

PEA : palmitoyl-ethanolamine

PI3-K : phosphatidyl-inositol 3-kinases

PLA1 : phospholipase A1

PLC : phospholipase C

POMC : pro-opiomelanocortin

PPAR-α : peroxisome proliferator-activated receptor alpha

PR : progesterone receptors

PVN : paraventricular nucleus

PYY : peptide YY

Q1 : first quadrupole

Q2 : second quadrupole

Q3 : third quadrupole

QC : quality control

RF : radiofrequency

RIA : radioimmunoassay

RYGB : Roux-en-Y gastric bypass

S/N : signal-noise ratio

SBP : systolic blood pressure

SCOUT : Sibutramine Cardiovascular Outcomes Trial

SER : smooth endoplasmic reticulum

SHBG : sex hormone-binding globulin

SPE : solid phase extraction

SRM : single reaction monitoring

ST : stubbies

T2DM : type II diabetes

THC : Δ^9-tetrahydrocannabinol

TOF : time of flight

TSH : thyroid-stimulating hormone

WAT : white adipose tissue

WHO : World Health Organization

INTRODUCTION

Obesity can be defined as an excess of body weight due to an imbalance between food intake and energy expenditure[1], leading to an accumulation of white adipose tissue (WAT) with adipocytes hypertrophy and hyperplasia[2]. Nowadays, obesity represents one of the most common issues of the public welfare in the majority of developed countries, and epidemiologic data about the high incidence and prevalence of this pathological condition are alarming. Indeed, obesity was defined by the World Health Organization (WHO) as a *global epidemic*[1], and as much as 9,8% of the world population is currently obese[3]. Obesity is generally classified by the *body mass index* (BMI)[1], a simple calculation developed by a Belgian statistician, Adolphe Quetelet, in 1871: it represents the ratio between a subject's weight and his height squared (kg/m^2). The classification according to the BMI is therefore the following: under-weight: ≤ 18.0 kg/m^2; normo-weight: 18,1-25,0; over-weight: 25,1-30,0; obese: 30,1-35,0; severe obesity: > 35,1.

The BMI, however, does not take into account the precise fat distribution and for this reason other measurements such as the hip-waist ratio or body fat percentage are useful to clinically define the obesity grade. The excess of body weight is associated in general with a reduction of life expectancy[4] since it leads to several pathologies, such as diabetes mellitus type II, cardiovascular diseases, different types of cancer, obstructive sleep apnea and many more[4]. There are several causes leading to obesity: an imbalanced diet, sedentary life and genetic predisposition are all factors participating to body weight gain[5-6]. Particularly interesting is the opinion by which obesity can be a maladaptation to environmental stress, which would lead to an imbalance of the neuro-endocrine axis[7]. Although imbalances of several hormonal systems have been described as critical in the pathophysiology of obesity, it is still unclear which alteration represents a cause and which an effect.

The identification of precise circulating biomarkers of obesity and their involvement in its pathophysiology could be essential to early diagnose potential co-morbidities and especially to better address the development of future therapeutic strategies. Currently, it is well known that several hormonal signals, such as for instance leptin[8], ghrelin[9] or adiponectin[8] among others, are altered in obese subjects. Some of these changes are secondary to obesity, while others could play an important role in its pathogenesis. Furthermore, published evidence also shows that other molecules, such as endocannabinoids (ECs) or steroid hormones might have a role in the physiopathology of obesity[10-11]. However, while alterations of several hormones have been well characterized in the obese population, a precise and reliable quantification of steroid hormones, in particular the androgens sub-group, and of the ECs is still lacking. Indeed, in most cases, it is not easy to perform an accurate measurement of these molecules: often, these analytes circulate at very low concentrations and their intra-group different chemical natures make the development of multi-analytes quantification methods an analytical challenge.

The most significant revolution in the recent history of analytical chemistry concerning quantification of bio-molecules is represented by the development of techniques based on mass spectrometry. These new

analytical tools allow us to have reliable and trustworthy results essential for both clinical analyses and scientific research, representing an authentic turning point for the entire scientific progress. In the last twenty years, the development of chromatography coupled with mass spectrometry is making possible the quantification of an increasing number of different molecules, which are not suitable to be measured with traditional techniques, such as for instance immunoassays[12]. This new technique joins the accurate separation of biological matrices components typical of chromatography with the specific identification and quantification of each molecule performed by the mass spectrometry[13]. Initially, quantification methods were developed using gas chromatography coupled with mass spectrometry (GC/MS); more recently, the quantification of a wide range of chemically different molecules has been made possible by coupling the mass spectrometry with the more versatile liquid chromatography[13]. The advent of these new technical devices performing accurate, sensitive and rapid analyses, has allowed discovering new markers, screening entire populations and increasing the number of molecules that can be measured and related with different pathophysiological mechanisms. The discovery or the better characterization of circulating biomarkers could lead, among others, to important progress in the field of obesity research.

In this context, I have been interested in improving the quantification of two types of circulating markers involved in obesity: steroid hormones and ECs, since the unavailability of sensitive and specific quantitative methods for these two classes of compounds has limited their investigation. My thesis will be therefore divided in two parts, to illustrate the studies that I carried out with chromatography tandem mass spectrometry for the measurement of these two groups of molecules.

In the first part of the present thesis (chapter two), which was carried out at the Center of Applied Biomedical Research (CRBA) in Bologna, we developed a sensitive, specific and accurate quantification method for nine steroid hormones using a liquid chromatography tandem mass spectrometry (LC-MS/MS) system. After the validation, the developed method was used first for a comparative study with immunometric methods, routinely used in the Laboratorio Centralizzato of the Policlinico Sant'Orsola Malpighi in Bologna to measure circulating steroids in patients. After the correlation study, we performed steroid measurements in a healthy population (179 males and 177 females), redefining circulating reference intervals. Indeed, steroids circulating reference intervals that have been used so far were established more than 50 years ago using radioimmunoassays (RIA); since our correlation study highlighted several limitations of this quantitative method, a redefinition of the reference intervals was needed. Afterwards, to test the involvement of steroid hormones in obesity, the developed method was used to define differences in circulating steroid hormones concentrations in three populations: normo-weight subjects (BMI: 18,1 – 25,0), over-weight subjects (BMI: 25,1 – 30,0) and obese subjects (BMI≥ 30,1). Indeed, among other alterations, visceral obesity, which is characterized by the accumulation of adipose mass in the visceral cavity, is associated with a hyper-activation of the *hypothalamic-pituitary-adrenal* axis (HPA)

and the impairment of the *hypothalamic-pituitary-gonadal* axis (HPG), leading to an altered production of steroid hormones, in particular glucocorticoids and androgens[10]. Our study had therefore as aim to better elucidate the real role of these molecular alterations in obesity.

In the second part of the project (chapter three), which was developed at the Neurocentre Magendie INSERM U862 in Bordeaux, we evaluated another class of circulating molecules whose levels are altered in obese subjects: the ECs. In the last twenty years, the relationship between ECs and obesity has been object of extensive research[11-14]. Since the beginnings of 90s an increasing number of studies have attempted to clarify the involvement of the endocannabinoid system (ECS) in the context of food intake and energy balance[14]. While, however, alterations of the ECS in obesity animal models have been extensively documented[15-16], the role of this system in the pathophysiology of human obesity is still poorly defined. The first methods to quantify ECs in plasma were developed almost twenty years ago, and an imbalance of these circulating molecules in humans was highlighted[17-19]. Nowadays we know that plasma ECs are higher in obese patients compared to normo-weight subjects and that these levels correlate with parameters related to obesity[19-20], despite the fact that the physiological role of these molecules is still unclear. Furthermore, it was shown that after a diet-induced weight loss, circulating ECs tend to decrease[21], further confirming that this alteration is strictly related to the obesity condition. In addition, it was also recently demonstrated that the plasma EC *anandamide* (AEA) levels are strongly related to the feeding status[22], increasing during fasting and decreasing after a meal, thus showing a profile of a meal initiator signal. Based on this evidence, we decided to carry out a project aimed at deifying alterations of circulating ECs in obese patients after a weight loss induced by bariatric surgery, currently the most effective long-term treatment for obesity. We measured basal and dynamic ECs plasma levels in 12 patients with severe obesity (BMI > 40 kg/m2 or BMI > 35 kg/m2 with obesity associated complications) before, one month after and six months after the Roux-en-Y gastric bypass (RYGB) intervention, currently one of the most performed types of bariatric surgery[23].

Moreover, to assess the recovery of circulating normal levels after a weight loss and a potential link with the ECS, we measured also androgens, the group of steroids involved particularly in male sexual functions, in our male patients undergoing RYGB. Indeed, data from animal studies have showed a link between the ECS and androgens, including the presence of the ECS on all the three levels of the HPG and the involvement of both ECs and androgens in the same biological functions, such as for instance reproduction[24].

In particular, we measured testosterone and dehydroepiandrosterone (DHEA) to assess whether after RYGB and the consequent weight loss, the HPG functionality and normal circulating levels of androgens are recovered. The eventual changes of circulating ECs and androgens could be then correlated to anthropometric parameters, such as the BMI, and between each other to determine if these two plasma

molecules altered by obesity can have a direct relationship. In humans, this link has never been tested, especially in relation to this pathology.

All together the findings illustrated in this thesis project will help better define the role of steroid hormones, in particular the androgen sub-group, and ECs in the framework of obesity in humans and the role that each type of molecule might have in its pathophysiology.

1

INTRODUCTION TO LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

I. Chromatography

Ia. Historical Notes

"I decided to build a machine equivalent to an array of about 200 separating funnels".

(Archer John Porter Martin)

Chromatography is an analytical technique used to separate a complex matrix in single components, determining the separation on different characteristics of the molecules.

The origin of chromatography dates back to the late 19th century. The first "chromatography-like" technique is attributed to a Russian botanist, Mikhail Tsvet, that in 1890 had used a liquid-adsorption column containing calcium carbonate to separate plants components based on their different colors[25]: green for the chlorophyll, orange for the carotenes and yellow for the xanthophylls (figure 1.1), giving to this technique the name chromatography, literally "*color writing*".

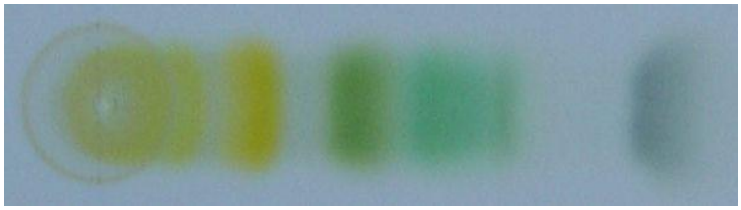


Figure 1.1: first chromatography developed by Tsvet.

In 1922 LS Palmer used the principles developed by Tsvet to separate carotenoids present in milk, butter and other matrices[26]. During the first half of the 20th century, the separation principle based on different characteristics of the material components was used to analyze the composition of other natural products. In 1943 Archer John Porter Martin and Richard Laurence Millington Synge developed the *partition chromatography* that was used for the first time to separate different amino-acids[27], obtaining for this innovation the Nobel Prize in chemistry in 1952. Since then, the technology advanced rapidly. In 1947, Fritz

and Cremer developed the first type of *gas chromatography*[28] (GC), characterized by a gaseous mobile phase, while the first *high pressure liquid chromatography* (HPLC), using a liquid solution as mobile phase, was built by Csaba Horvath in 1970, achieving the analytical separation also for biological compounds which could not be vaporized. After that, HPLC quickly improved with the development of new column packing materials. The history of chromatography is represented in figure 1.2.

Nowadays chromatography is an indispensable analytical technology used worldwide in every branch of physical and biological science to separate chemical compounds for any type of qualitative and quantitative analysis. This technique is constantly improving, allowing the separation of increasingly greater number of molecules.

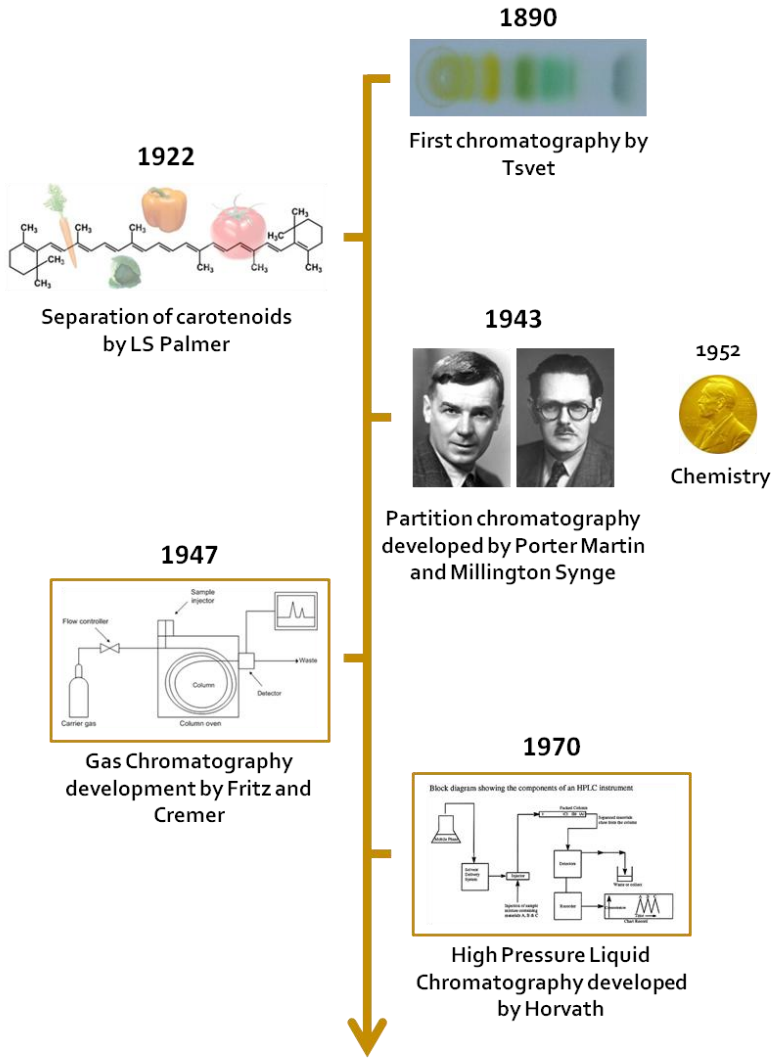


Figure 1.2: Chromatography time-line.

Ib. Technical Notes

The separation of mixture constituents using chromatography is achieved by their different distribution between two different phases over time (figure 1.3). Every chromatographic system is characterized by:

- *Stationary phase*: is the phase immobilized on the support particles, or on the inner wall of column tubing. Its interaction with the analytes determines the separation.
- *Mobile phase*: is the phase carrying the analytes through the stationary phase, moving in a specific direction. It can be a gas (GC) going through the stationary phase under pressure, or a liquid (*liquid chromatography*, LC) going through the stationary phase by gravity or using a pump system.

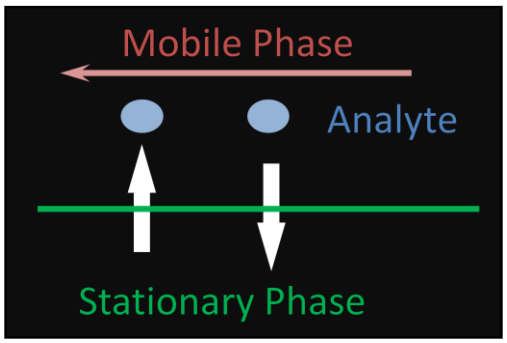


Figure 1.3: Analytes repartition between stationary and mobile phase.

The choice of the suitable stationary and mobile phase depends on the physical-chemical proprieties of the analytes to separate.

There are different types of chromatography based on the attractive force used for the separation:

- *Normal phase*: for polar compounds. The stationary phase is polar while the mobile phase is non-polar. The separation is based on the analyte adsorption on the polar surface of the stationary phase.
- *Reversed phase*: for non-polar compounds. The stationary phase is non-polar while the mobile phase is polar. The separation is based on analyte partitioning between the mobile and stationary phase through hydrophobic interactions.
- *Ion Exchange*: for ionic compounds. The separation is based on ionic interactions of the analyte with the ionic group on the stationary phase.

- Size *Exclusion*: is a physical separation process. The stationary phase is a porous matrix and the separation is based on the different size and shape of analytes.

The result of a chromatographic analysis, as visual output, is the *chromatogram*, in which every *peak* corresponds to a different analyte. On the x-axis there is the time and on the y-axis there is the intensity of the signal obtained by the detector.

Ic. Chromatographic parameters

The chromatographic analysis performance is characterized by different parameters:

- *Retention time*: is the characteristic time that each analyte spends to pass through the chromatographic system under determinate conditions.
- *Capacity factor*: is the relative time that the analyte spends to go out from the column compared to an analyte that hasn't affinity for the stationary phase. This parameter is relevant because, unlike the retention time, it is independent of the column dimension and the mobile phase flow, so it can be used to compare analytes performance in different chromatographic systems.
- *Efficiency*: it evaluates peaks sharp shape and symmetry. Usually, it can be estimated with the "*theorical plate theory*". The plate model supposes that the chromatographic column contains a large number of separate layers, called *theoretical plates*. They represent the hypothetical zone in which the analyte establishes an equilibrium between stationary and mobile phase moving down the column from one plate to the next. Giving that, having many of them increases the efficacy of the separation.
- *Selectivity factor*: it is the difference in retention time of one molecular band relative to another. It is an indicator of the ability of the chromatographic system to separate two different analytes.
- *Resolution*: it shows the separation efficiency between two peaks in relation to their width. It is calculated as the ratio between the difference of retention time between two peaks and their width on average.

Id. High Pressure liquid chromatography

High pressure liquid chromatography is one of the most used analytical chemistry tool. It can have medical applications, detecting different analytes concentration in the blood; legal application, detecting drugs in urine; and it allows analyzing drugs composition and identifying components in complex mixtures. The HPLC technique is powerful and versatile and has many advantages compared to other types of chromatography, thanks to excellent reproducibility, high sensitivity, better resolution and furthermore it can be easily automatized.

The typical HPLC system consists of different devices (figure 1.4):

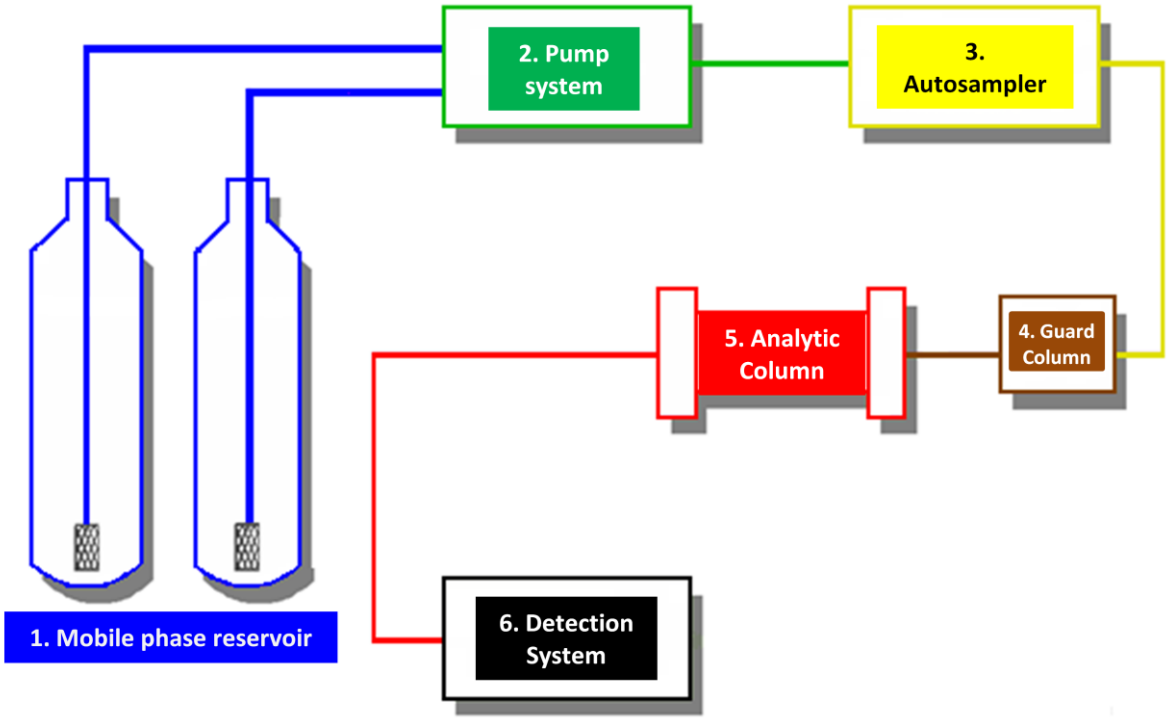


Figure 1.4: The HPLC system.

1. *Mobile phase reservoir*: the choice of the mobile phase depends on the required level of separation. Solvents should be of high purity and degassed, not corrosive, with a low viscosity and they must not alter analytes proprieties.

2. *Pump system*: this device is generating the mobile phase gradient. Depending on the analytes to separate, two types of gradients can be used: the isocratic gradient, in which mobile phase is constant during the overall chromatographic run, and the elution gradient, in which separation is achieved modulating the composition of mobile phase. The pump system must be able to work with very high pressure and to provide a constant and reproducible flow, guarantying the repeatability of analytes retention times.
3. *Autosampler*: the correct sample injection is a very important factor during a chromatographic analysis. The injection system has to provide injection volume repeatability and a good versatility in samples handling. The modern autosamplers use mostly *Rheodyne* injector valve technology (figure 1.5), to be simultaneously connected with all the HPLC devices, and a temperature controller for the samples tray.

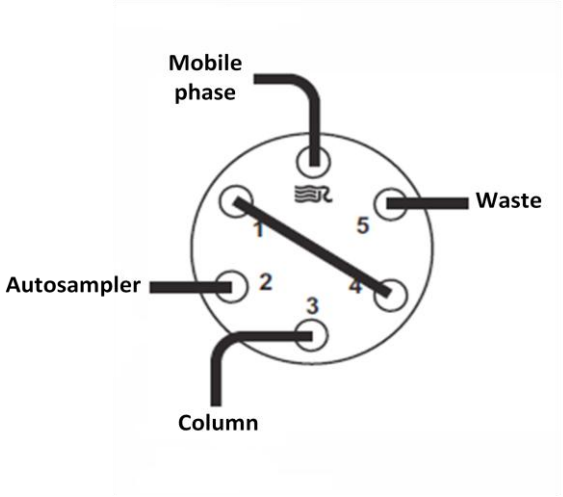


Figure 1.5: Rheodyne System.

4. *Guard column*: it is a very short column with the same characteristics of the analytic column (e.g. composition of stationary phase and diameter). The guard column is positioned between the injector and the analytic column and it is essential to restrain contaminant materials. It has to be replaced more often than the analytic column.
5. *Analytic column*: the column contains the stationary phase and it is the heart of every chromatographic system. Nowadays there are many types of column, which are different for the stationary phase composition, diameter and length; the pick depends on the analytes to separate. The column resolution power must be periodically evaluated: it is the main column life indicator.

6. *Detection system*: after the separation, the chromatographic system needs a detector to reveal the nature of the analytes. The detector must have high sensitivity, since the analytes volume after the separation is usually very small; it has to be fast and it has to give quantitative and qualitative interpretations. The most common revelation systems are UV detectors and mass spectrometers, their use depends on the nature of the analyzed compounds.

II. Mass spectrometry

IIa. Historical Notes

"The future of mass spectrometry looks to be as exciting as its past"

(Jennifer Griffiths, "*A brief history of mass spectrometry*", 2008)

The mass spectrometry analytical technique has origin in the physics field. In 1897 J.J. Thomson and his laboratory assistant E. Everett developed the first "spectrometer-like" apparatus to measure particles mass-to-charge ratio. While they were studying the transmission of electricity through gases, they identified the first subatomic particles: the electron[29]. For this great discovery, Thomson received in 1906 the Nobel Prize in physics. Afterwards, in 1919, Francis Aston built what later was recognized as the first authentic mass spectrometer, using it to measure masses of charged atoms. In the first half of the 20th century Aston improved the resolution power of his instrument and used it to identify the elemental isotopes[30], receiving the Nobel Prize in physics in 1922. In the same half of the century, Alfred Nier, an electrical engineer, built several revolutionary instruments[31], and promoting the use of this technique to scientists outside the restricted physicists community. In the 1940s, mass spectrometers were commercially available, but at that time, their use was limited to quantitative analysis. A group of three chemists, Fred McLafferty, Klaus Biemann and Carl Djerassi, through systematic mass spectrometry experiments, noticed that every class of organic molecules had a different and specific fragmentation pattern[29]. In this way, they could determine the structures of unknown molecules, placing the basis of the current qualitative-quantitative use of mass spectrometry. In 1946 William Stephens presented the concept of the mass spectrometer *"time of flight"* (TOF)[32]. At the beginnings of 1980s small organic molecules were routinely analyzed, but not the macromolecules, more difficult to ionize. A big turning point for this issue was in the 1984, when Franz Hillenkamp and Michael Karas developed the *"matrix-assisted laser desorption/ionization"* (MALDI)[33] building up a ionization technique also for macromolecules, and simultaneously John Fenn developed the "*electrospray ionization"* (ESI)[34], receiving for this technical discovery the Chemistry Nobel Prize in 2002. The mass spectrometry main historical events are represented in figure 1.6.

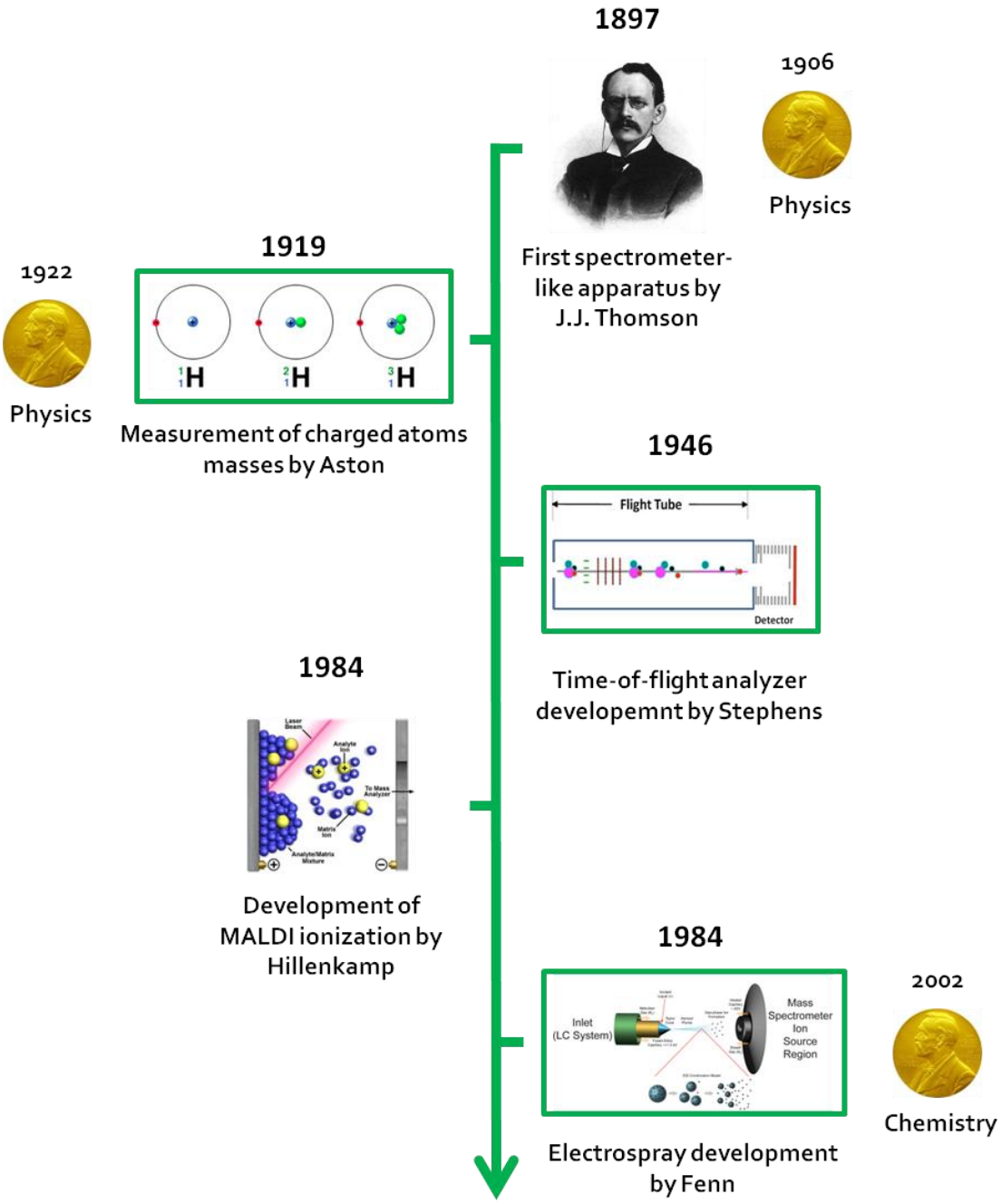


Figure 1.6: Mass spectrometry time-line

IIb. Technical Notes

The mass spectrometer is an analytical technique used to measure the mass of charged molecules (ions) evaluating their *mass-to-charge* ratio (*m/z*), generally using electromagnetic fields. It is a key analytic tool for qualitative analysis, elucidating molecular structures, and for quantitative analysis, determining the amount of unknown compounds.

When the sample is loaded into the mass spectrometer, its molecules are ionized usually through the expulsion ("*negative mode*") or the gain ("*positive mode*") of a proton and then separated according to their m/z. Afterwards, the ions are detected and the visual output obtained is a *mass spectrum*, where on the x-axis there is the m/z and on the y-axis there is the relative intensity of each ion. Furthermore, to improve the analysis specificity, modern mass spectrometers use a "double" selection of charged ions, and this highly specific technique is called *tandem mass* (MS/MS). After the ionization, there is a first analyzer in which the ions (*precursor ions*) are selected according to their m/z (MS analysis) followed by another spectrometer region, called "collision chamber", in which the ions collide with an inert gas breaking their original structure in some smaller ions, called *product ions*. After the collision chamber there is another analyzer, similar to the first one, that selects the product ions according to their m/z (second MS analysis). In this way, the specificity is extremely high because there are three levels of selection: the precursor ion m/z, the fragmentation pattern, distinctive for each molecule, and the product ion m/z. The fragmentation pattern is used especially in qualitative analysis, because breaking molecules can give important information about the compound structure.

IIc. Structure of a mass spectrometer

Almost all the mass spectrometers have a recurring structure. They are mainly constituted by an ionization source, an analyzer and a detector (figure 1.7).

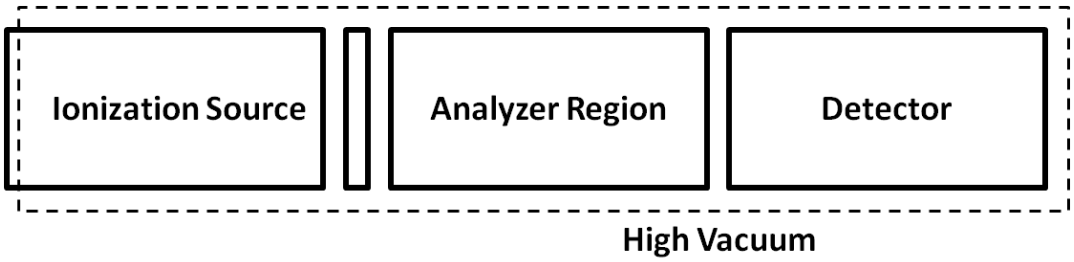


Figure 1.7: Structure of a mass spectrometer.

i. The ionization source

The source is the key component of a mass spectrometer: here takes place the ionization, the basic process of every mass spectrometry analysis. There are different kinds of ionization techniques: some of them are "*hard ionization*", using high energy and high power, often causing a partial molecule fragmentation; other ionization techniques, mainly used, are defined "*soft ionization*", since they involve a smaller amount of energy. Among all the soft ionization techniques, the most used are the *electrospray ionization* (ESI) and the *atmospheric pressure chemical ionization* (APCI) for liquid samples (e.g. mass spectrometry coupled with HPLC or micro-injector); the choice depends on the molecule type to analyze (figure 1.8). Furthermore, the MALDI ionization technique is the only one suitable for analytes with a very high molecular weight (>100.000 Da), like peptides and polymers.

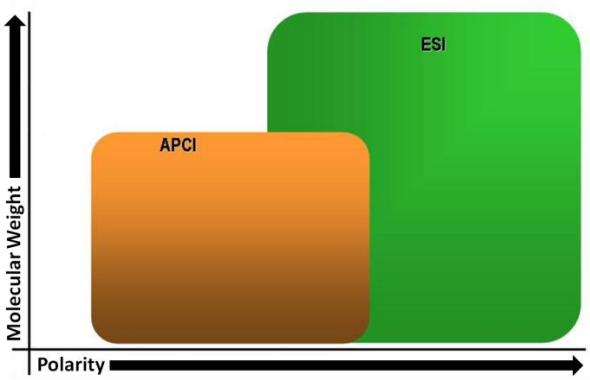


Figure 1.8: Ionization type suitability according to analytes polarity and molecular weight.

1. The electrospray ionization, ESI

ESI is probably the most diffused ionization technique, since it is suitable for a large number of molecules. It is a soft ionization used for analytes with a medium – high polarity and a molecular weight up to 150.000 Da. Usually, the sample is dissolved in a mixture of water and organic solvent, such as methanol or acetonitrile, and introduced in an high-voltage capillary (that can be heated to facilitate the solvent evaporation) and then nebulized with the help of an inert gas, for instance nitrogen or carbon dioxide. The nebulization allows the formation of charged droplets in the first vacuum region of the spectrometer, where the solvent evaporates and the droplets form a charged jet (through a process called *Coulomb fission*) that is carried in the mass spectrometer analyzer (figure 1.9). This ionization technique is perfectly compatible with an HPLC system and has some advantages as the possibility to analyze macromolecules and a good sensitivity. The disadvantages are the incompatibility with saline solutions and high HPLC flow: for a good performance the HPLC flow should be < 0,5 ml/min. There could be also some issues related to the detection of the exact mass of the molecules because of the formation of *adducts* (the precursor ion can bind sodium molecules during the ionization process) or *multiply charged ions*; this can however be avoided by adding a small percentage of weak acid (e.g. acetic acid) to the HPLC mobile phase.

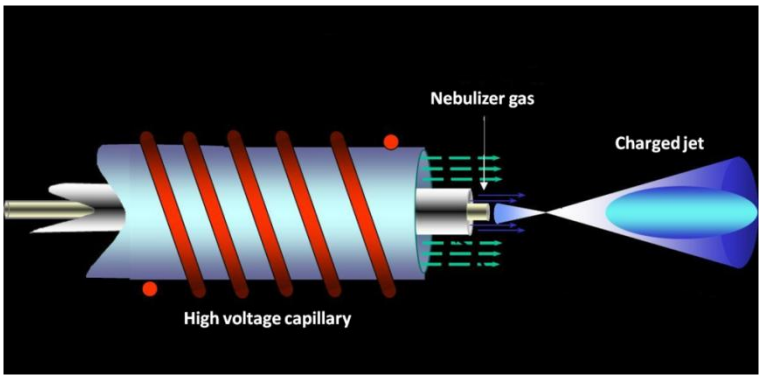


Figure 1.9: ESI ionization.

2. The Atmospheric Pressure Chemical Ionization, APCI

APCI is the best ionization technique when working with high HPLC flow (typically between 0.5 and 2 ml/min) and it is suitable for analytes having low molecular weight (<1000 Da) and low polarity. It is a soft ionization technique, but it has a stronger fragmentation power than ESI. The eluting analyte is carried through a capillary from the chromatographic system to a heated nebulizer needle, typically working at very high temperatures (usually >400°C), and it is sprayed with a high-flow rate of nitrogen (*chemical ionization*). The solvent evaporates and the spray is then subjected to a high potential corona discharge needle that creates ions (figure 1.10). With this type of ionization is very rare to find adducts or multiply charged ions.

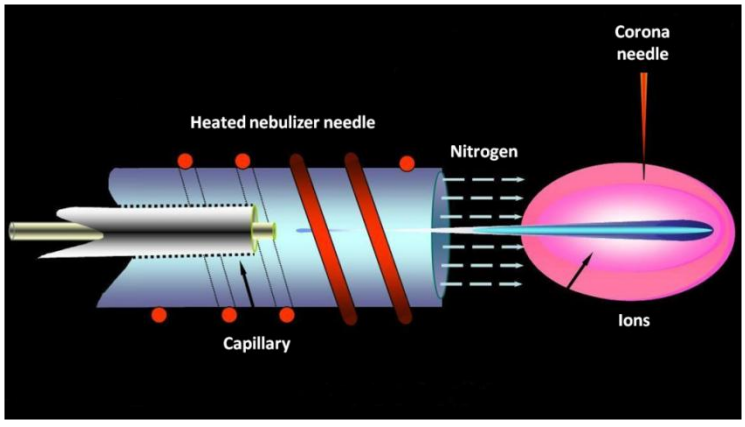


Figure 1.10: APCI ionization.

ii. The mass analyzer

The mass analyzer is the spectrometer region in which the ions coming from the source are sorted and separated according to their m/z ratio. There are several parameters that can be used to evaluate a mass analyzer:

- The *mass resolution power*: the measure of the analyzer ability to discriminate two peaks of slightly different m/z.
- The *mass range*: the range of *m/z* measured by the analyzer.
- The *mass accuracy*: the ratio between the *m/z* measurement obtained by the analyzer and the true analytes m/z.

Among different types of mass analyzer, the *quadrupole mass filter* and the *TOF* are definitely the most diffused. TOF analyzer is usually coupled with MALDI ionization source; the MALDI-TOF system allows analyzing high masses over 1000 KDa. Ion m/z is determined via a time measurement: ions are accelerated in a flight tube and the time spent by each ion to reach the detector, depending on the ion m/z, is measured.

Almost all mass spectrometry instruments coupled with a chromatographic system use the quadrupole as mass analyzer. Indeed, it is completely compatible with ESI and APCI ionizations and it is the easiest analyzer to use with a wide range of applicability and high specificity.

The quadrupole is basically constituted by four parallel metal rods (length approximately of 20 cm), which define the ions path using oscillating electrical fields, stabilizing or destabilizing them through a radiofrequency (RF) (figure 1.11). This way, only the ions in the selected m/z range can pass through the system and arrive to the detector; other ions with a non corresponding m/z have unstable trajectories and will collide with the rods.

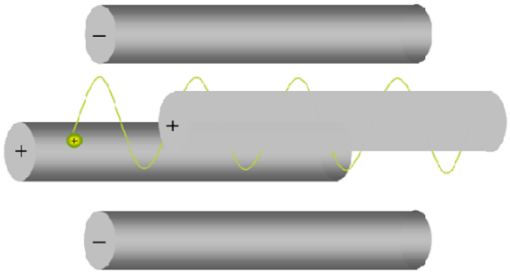


Figure 1.11: Typical quadrupole structure.

A common variation of the classic single quadrupole instrument is the *triple quadrupole*. These instruments have three linear quadrupole stages: the first quadrupole (Q1) acts as a mass filter to sort and let pass the *precursor ions* in the selected m/z range; the second quadrupole (Q2) is a collision chamber in which ions collide with an inert gas, breaking their original structure and producing specific fragments also called *product ions*; the third quadrupole (Q3), equal to the first, transmits to the detector product ions in the selected m/z range (figure 1.12). Frequently, between the ionization source and Q1, there is another quadrupole (Q0) that is not acting as a mass filter, but is only stabilizing the ions trajectories.

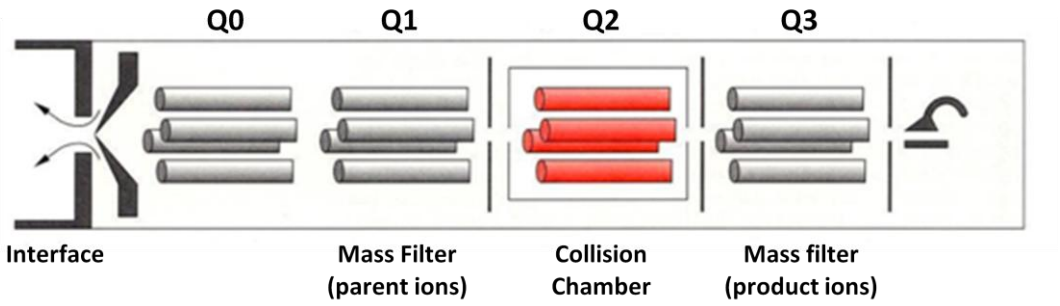


Figure 1.12: Triple quadrupole arrangement.

There are two MS/MS operative modes:

- *Single reaction monitoring* (SRM): both mass analyzers (Q1 and Q3) are set to a selected mass. This is a very selective analysis mode.

- *Multiple reaction monitoring* (MRM): in this analysis mode the operator can sort more than one product ion (usually two) with the Q3 increasing the specificity of the analysis (figure 1.13).

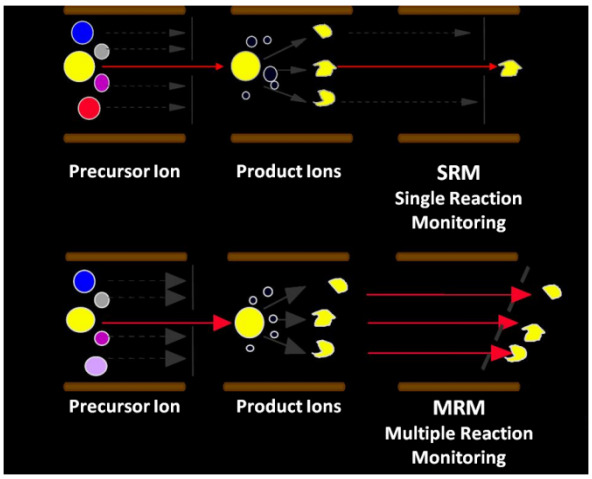


Figure 1.13: SRM and MRM operative modes.

iii. The detector

A detector, the final part of a mass spectrometry instrument, must identify and quantify ion signals. In large part of mass spectrometry experiments, there is a small ions amount reaching the detector, so it is necessary to amplify the signal. The most commonly used detector is an *electron multiplier*, a vacuum tube in which the incident charges signal is multiplied through a series of sequential electrodes called *dynodes*. The result is a huge signal amplification that can be elaborated by the calculator obtaining the classical *mass spectrum*.

III. Liquid chromatography tandem mass spectrometry

IIIa. Historical and Technical Notes

"The next time that you run an LC-MS instrument, or sit down with others to review some LC-MS data, just spend a short time and reflect on all the groundbreaking work and all the heartache that went into the development of this technique by a few pioneers who had the dogged resolve to overcome what was seen at the time to be an insolvable problem."

(Frank Pullen, "The fascinating history of the development of LC-MS; a personal perspective", 2010)

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a technique that combines the advantages of two complementary methods: the separation skills of liquid chromatography, which can fractionate complex samples in single analytes, with the mass analysis capabilities of mass spectrometry, which can precisely measure or accurately identify small analytes amount. From the historical point of view, LC-MS/MS development was not easy: the problem was to combine two techniques of which the former works with high liquids volume and pressure, and the latter works with high vacuum (approximately 10^{-7} torr in the source). In 1968 the first kind of *interface* between the two instruments was proposed by a Russian scientist, Victor Tal'Roze, who tried to spray a very small amount of liquid into a conventional mass spectrometer[35] with a simple approach. In 1974 Baldwin and MacLaferty tried the same experiment but increasing the liquid amount[36], developing a DLI LC-MS (*direct liquid injection liquid chromatography mass spectrometry*), but the amount of liquid efficiently ionized was still too small. In 1975 three scientists, Horning, Dziric and Carroll, developed the first *atmospheric pressure ionization* (API)[37]; however the spectra produced with this ionization were not so clear for the presence of cluster ions[35]. In 1980 Vestal and Blackley built up the first *Thermospray interface*, which allowed the direct liquid ionization also for low volatile molecules, but this approach was still presenting some limits as the necessity to have ammonium acetate in the mobile phase to drive the ionization[38]. The real decisive moment was in the first half of 1980s, when the APCI ionization source and the ESI ionization source were developed, coupling finally together the liquid chromatography and the mass spectrometry without limits of mobile phase composition or analytes chemical characteristics. The APCI was developed by Bruce Thomson[39], who was working in the mass spectrometry company *Sciex*, while the ESI was developed by John Fenn at Yale University[34], as mentioned before. Afterwards, LC-MS/MS improved rapidly thanks to new commercially available instruments, better pre-analytic techniques and new materials for LC columns stationary phases.

The LC-MS/MS development represents a turning point in the analytical chemistry history. Since its technical features and the quantitative-qualitative analysis spectrum, it can offer an endless range of applications, as for instance newborns screening (in particular for metabolic disorders), drug composition analysis or development, pharmacokinetic studies, bimolecular analysis, protein identification, forensic medicine, doping detection in sport, and many more. Nowadays this technique represents one of the most diffused within analytical chemistry laboratories. The history of LC/MS is represented in figure 1.14.

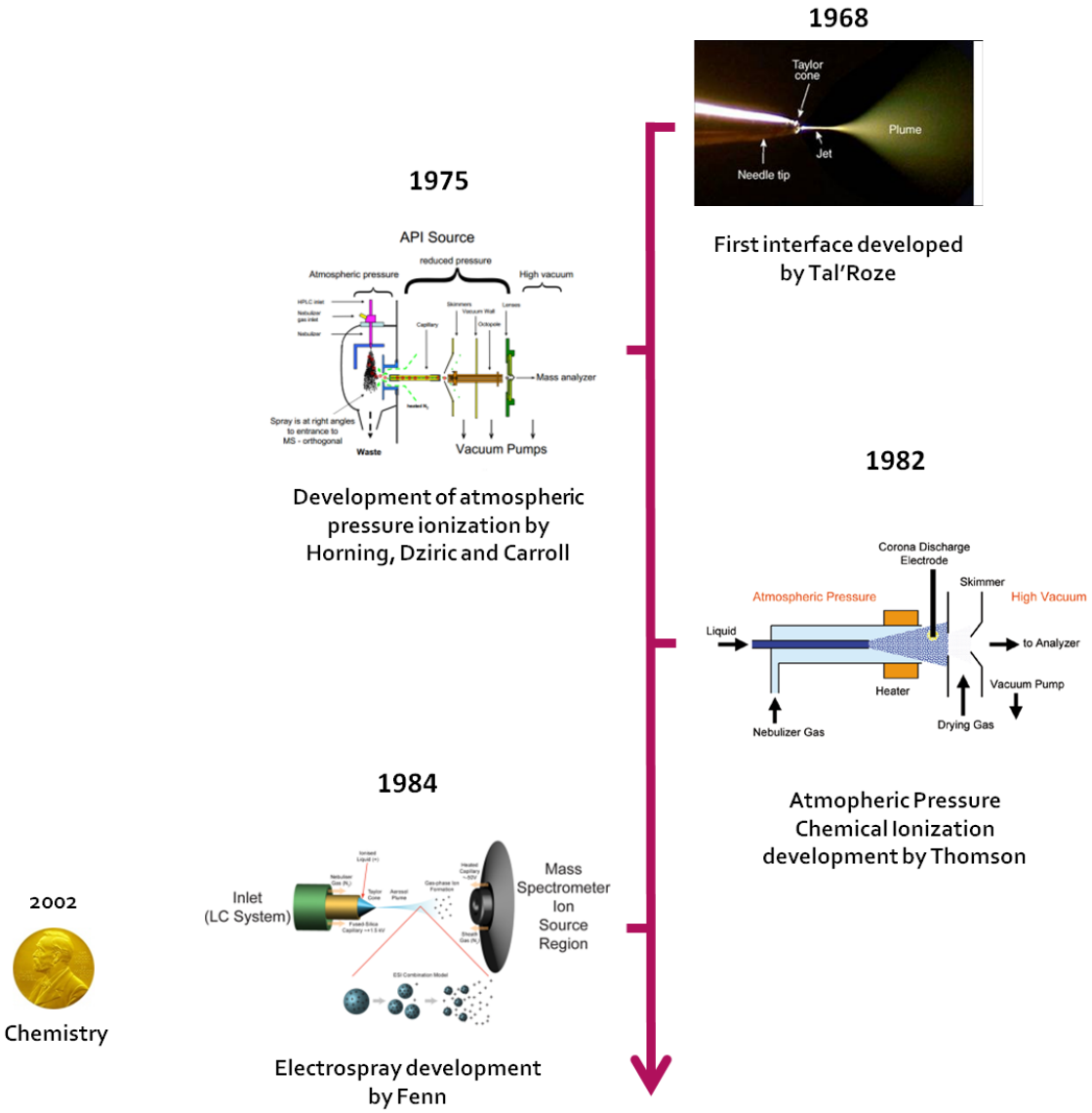


Figure 1.14: LC/MS time-line.

2

CIRCULATING STEROID HORMONES MEASUREMENT IN HEALTHY AND OBESE PATIENTS

Quantitative circulating molecules measurements have always been an important tool for endocrinology. The development of the first RIA aimed at insulin measurement in the 50s allowed for the first time the measurement of a hormone in the blood[40]. Soon after insulin, quantitative techniques based on RIA were developed for steroid hormones[13]. This wide hormone class has been always extensively studied since steroid hormones are involved in many physiological and pathological processes essential for the human body. The steroid class includes a great number of molecules, some of which have been very well characterized as for instance glucocorticoids or sexual steroids. The precise and accurate measure of circulating steroid hormones is essential for carrying out diagnoses and follow-up of many endocrine pathologies[13]. Nowadays, in the clinical practice, steroid hormones are quantified using immunoassays. This technique has a very good sensitivity, but the specificity is considered a limit, since it is critically depends on the antibody used. Indeed, many discrepancies among measurements carried out in different laboratories have been evidenced[12], especially for steroid hormones circulating with very low concentrations, such as testosterone in women[41]. With the advent of GC/MS, new quantification methods for steroids were developed, increasingly sensitive and specific giving accurate and precise results. The limit of GC/MS is the derivatization step: steroids are not volatile molecules and consequently they are not suitable to be analyzed with this technique; to elude this problem, analytes moieties are chemically modified to make compounds more volatile and thermostable. This step has been considered the limit of this technique because the pre-analytical sample preparation becomes laborious and time-consuming[42], making complicated the introduction of the GC/MS as routine technique in clinical laboratories.

The introduction of LC-MS/MS in endocrinology has been considered an epochal revolution. This technique put together the RIA practicality and the GC/MS reliable technical features, providing fast and effortless multi-analyte measurements[13]. In the last years, always more quantitative multi-analytes methods with this technique have been developed for several molecules, and also for steroid hormones[43-44].

In this part of the thesis, carried out at the Center for Applied Biomedical Research (CRBA) in Bologna we chose this technique to develop a sensitive, accurate and specific method for the quantification of nine steroid hormones: cortisol, corticosterone, 11-deoxycortisol, androstenedione, deoxycorticosterone (DOC), testosterone, 17OH-progesterone, DHEA and progesterone. These steroids were chosen for their biological relevance and their involvement in several pathological states but also for chemical common characteristics, thus allowing the development of a fast multi-analytes technique.

This method has been validated following the Food and Drug Administration (FDA)[45] guidelines for the validation of bioanalytical methods. After the validation we used this method to carry out a comparative study with immunoassay, currently used at the Laboratorio Centralizzato of the Policlinico Sant'Orsola Malpighi in Bologna to quantify these molecules.

The reference intervals in healthy population for steroid hormones were defined in the 50s performing a healthy population sera screening with RIA. Since reference intervals are fundamental to diagnose pathologies and to establish diagnosis criteria, and since our comparative study evidenced the already well known limits of immunoassays, a review of these normality ranges was urgently needed. We measured the nine steroid hormones included in our analytical method in 179 healthy males and 177 healthy females to redefine their circulating normal range with LC-MS/MS.

Once established new steroids reference intervals in healthy subjects, we decided to measure these analytes in over-weight and obese patients. Obesity is a worldwide diffused pathological condition in which there is an imbalance between food intake and energy expenditure. This pathology has been intensely studied and an imbalance of several different physiological endocrine systems has been found. As well as other endocrine axes, steroids production and functionalities are altered in obesity, in particular the HPA axis and the HPG axis. The impairment of these two axes leads to important changes in circulating concentrations of some steroid hormones: glucocorticoids concentrations result typically increased and sex steroids are altered in a sex dependent manner[10]. Since among the steroids included in our analytical method there are some of the principal glucocorticoids (cortisol and 11-deoxycortisol) and androgens (testosterone, DHEA and androstenedione) and also an intermediate of both glucocorticoids and androgens synthesis (17OHprogesterone), we carried out these measurements to obtain circulating profiles in subjects with different BMI and verify the alterations of circulating levels in these subjects.

The precise quantitative assessment of steroid hormones imbalance in obese patients can help to elucidate the mechanisms underlying these altered concentrations, since they are still poorly understood.

I. Steroid Hormones

Ia. Biosynthesis and Transport

Steroid hormones are a wide hormone class including a large number of molecules. These hormones have a key role in body physiology, growth and energy balance, regulating many basic processes[46].

In general, all the steroid hormones have a very similar chemical structure; indeed, they are all derived from cholesterol, from which they take the characteristic basic structure with four *cicloalkane* rings (three cyclohexan and one cyclopentan) connected to each other (figure 2.1).

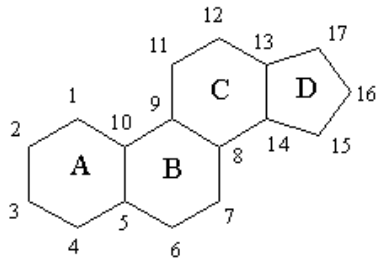


Figure 2.1: Characteristic steroids structure, the cicloalkane rings.

The different functional groups linked to the cicloalkane rings structure confer to every steroid the characteristic biological proprieties. The *steroidogenesis*, the biological process by which all the steroid hormones are generated from the cholesterol, is a complex multistep cascade regulated by a large number of different enzymes[47] (figure 2.2) located in mitochondria and smooth endoplasmic reticulum (SER). The *cytochrome P450c11a1*, located in mitochondria, catalyses the first, slower and therefore rate limiting step for the whole steroidogenesis cascade: the conversion of cholesterol in pregnenolone[47].

These hormones are synthesized mostly by the adrenal cortex, testicles, ovary and brain (*neurosteroids*) but a small part is produced also in some peripheral tissues, like the adipose tissue. Steroid hormones can be divided in three main functional classes: *mineralocorticoids* produced in the *zona glomerulosa,* the outermost layer of the adrenal cortex, *glucocorticoids*, produced in the *zona fascicolata* of the adrenal cortex and *sexual hormones,* produced mainly by testicles and *zona reticularis* of the adrenal cortex (*androgens*) and ovary (*estrogens*)[46] (figure 2.3).

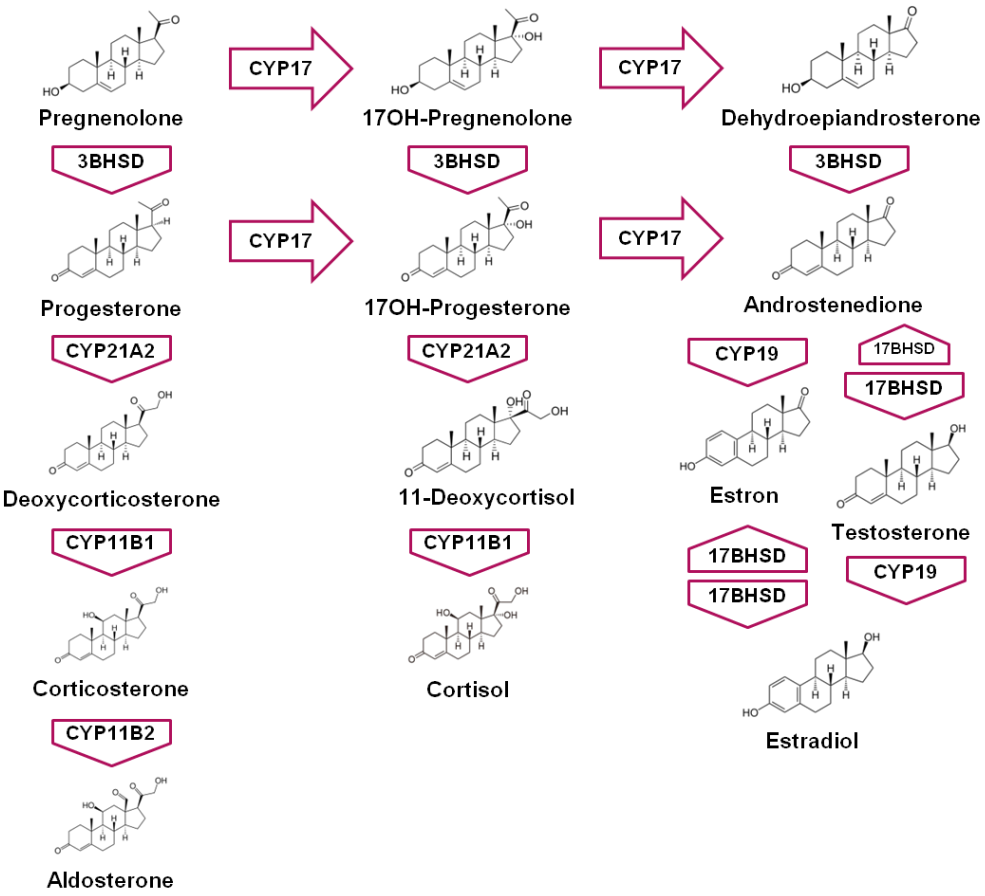


Figure 2.2: Steroidogenesis.

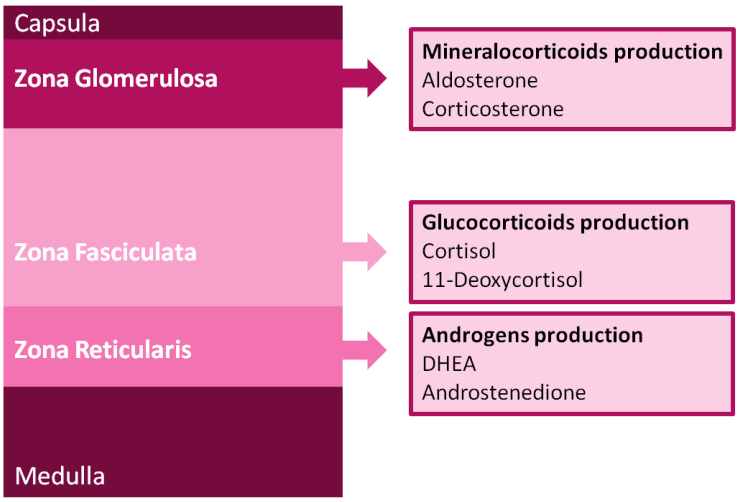


Figure 2.3: Adrenal layers and relative steroids production.

Steroid hormones biosynthesis is not directly dependent on transcription: the regulation of steroidogenesis involves the control of enzymes which modify cholesterol in the steroid hormone of interest and the production of single hormones is stimulated and regulated by other hormones upstream, as for instance *adrenocorticotropic hormone* (ACTH) for cortisol synthesis and *follicle-stimulating hormone* (FSH) for estradiol synthesis[48].

Unlike peptide hormones, steroid hormones are not stored but secreted as soon as they are produced and their secretion rate is directly dependent on the synthesis. In various biological fluids they exist at three different states: free, bound to transport proteins and conjugates. The free fraction, the less abundant in the blood (1-8%), represents the biologically active fraction. Because of their lipophilic nature, they are generally carried in the blood bound to specific glycoprotein such as albumin or the more specific *sex hormone-binding globulin* (SHBG), carriers for sexual steroids, or *corticosteroid-binding globulin* (CBG); furthermore, this fraction represents a "circulating reserve" of these hormones, providing free active hormones when the production rate decreases. Lastly, the conjugated part is the fraction linked to hydrophilic moieties, to facilitate their excretion through the liver and kidneys[48].

Ib. Receptors and genomic effects

Since their lipophilic nature, steroid hormones can easily enter in the cytosol trough the plasma membrane, binding intracellular or nuclear receptors, expressed only in target tissues[49]. The structural organization of steroids receptors is well preserved, consisting of four distinct parts (figure 2.4): a variable domain that can interact with other transcription factors (1) at the NH_2 terminal, DNA *zinc finger* binding domain (2), a dimerization domain (3) and the hormone binding domain (4) at the COOH terminal, which is different in every type of steroids receptor. Once the steroid binds the specific receptor, it causes a conformational change that allows the receptor to bind highly specific DNA sequences, called *hormone responsive elements* (HRE)[49]. The HRE sequences are very similar in every tissue for length and structure, but they have different hormone binding region depending on the target steroid they can bind and the target tissue in which they are expressed. The hormone-receptor complex acts as a transcription factor, which regulates gene transcription[49]. After the transcription, the *messenger RNA* (mRNA) is translated into specific protein by ribosomes, carrying out functions in target cells (figure 2.5). This genomic steroid mediated effect is a "*long term effect*" because it is directly influencing proteins synthesis and therefore it is slower than other kinds of hormonal actions.

Figure 2.4 : Structural organization of a steroid receptor.

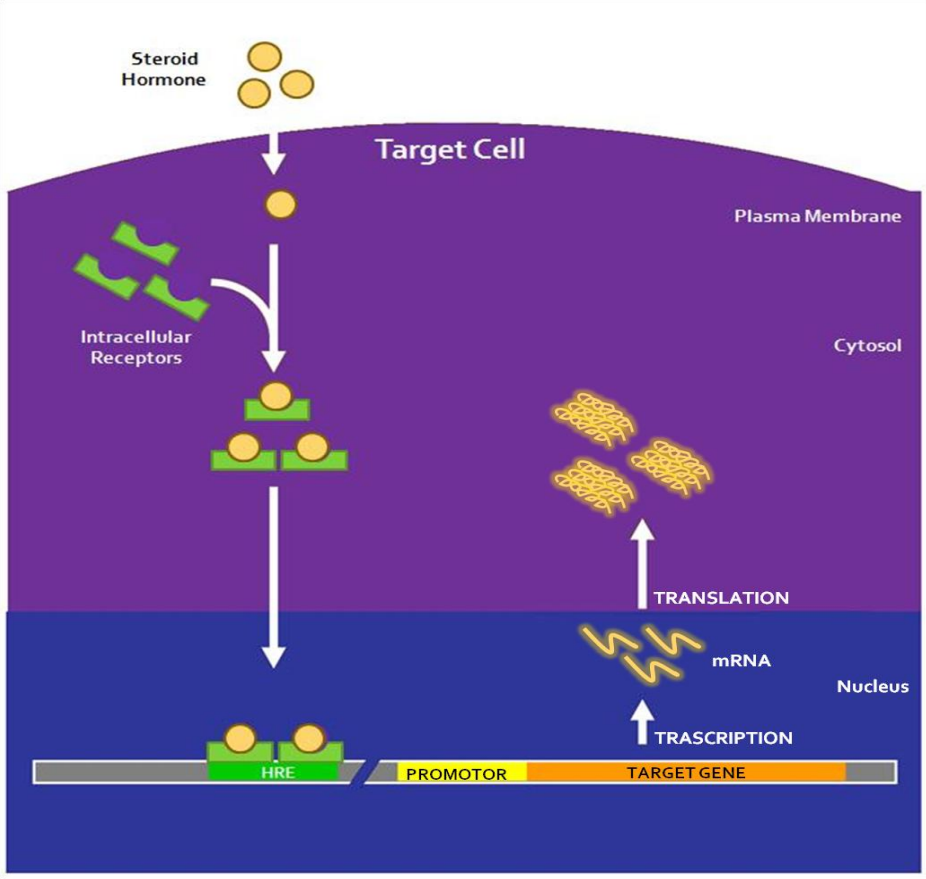


Figure 2.5 : Steroids receptor mechanism of action.

Ic. Physiology

As told before, steroid hormones can be grouped in different categories characterized by a wide spectrum of physiological actions.

1. Mineralocorticoids

The primary mineralocorticoids function is the control of salt-water balance. The most important hormone in this category is the **aldosterone**, which plays an important role maintaining extracellular fluid volume by conserving body sodium.

Aldosterone is produced in the zona glomerulosa of the adrenal glands by the enzyme *CYP11B2*, after the conversion of the C18 methyl group of the *corticosterone*, another mineralocorticoid hormone, in an aldehyde. Aldosterone is largely secreted in response to signals arising from the kidney when a reduction in circulating fluid volume is detected. The principal aldosterone regulator is the *renin-angiotensin system*: hypovolemia or hypotension sensed by the kidneys stimulates the justaglomerulal cells to produce *renin*; renin acts on *angiotensin*, a protein secreted by the liver, to form *angiotensin I* which is further cleaved by *angiotensin converting enzyme* (ACE), secreted by the lungs, to *angiotensin II*, a powerful vasoconstrictor, which lastly acts on the adrenal zona glomerulosa to stimulate aldosterone production[48] (figure 2.6). Aldosterone acts on kidneys to promote active re-absorption of sodium and passive reabsorption of water; furthermore, it provides excretion of potassium, which stimulates aldosterone biosynthesis, providing a feedback control mechanism. Viceversa, potassium low levels directly decrease aldosterone production.

Following the general mechanism described above, aldosterone binds the nuclear *mineralocorticoids receptors* (MR) in target cells, mainly in kidneys, causing transcriptional changes typical of steroid hormones action[50]. The physiological aldosterone concentration in the blood is very low and, like the majority of steroid hormones, its circulating concentration follows a physiological circadian rhythm: the highest concentration is around 8 a.m. and the lowest is around 11 p.m[48].

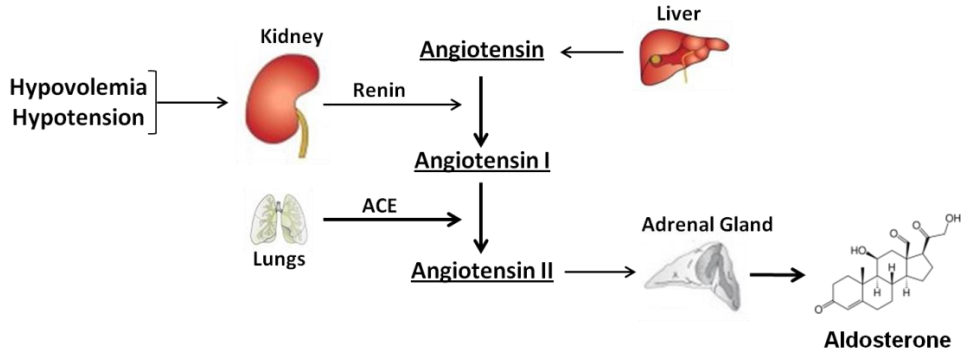


Figure 2.6: Aldosterone production induced by rennin-angiotensin system.

2. Glucocorticoids

Glucocorticoids are the steroid hormones in general responsible for the glucose metabolism, but they have several other different functions. The primary and most powerful hormone of this group is **cortisol**. Glucocorticoids synthesis is regulated by the HPA axis, mostly trough ACTH, a 39 amino acids peptide that is synthesized within the anterior pituitary gland from the 241 amino acids precursor called *pro-opiomelanocortin* (POMC), in response to the *corticotropin-releasing hormone* (CRH) produced by the *paraventricular nucleus of the hypothalamus* (PVN), mainly in response to stress situations[51]. ACTH acts on the zona fasciculata of the adrenal cortex inducing glucocorticoids production through ACTH receptors on the adrenal cells surface, stimulating the cholesterol delivery to mitochondria to start the steroidogenesis. As told before, glucocorticoids are responsible for a wide range of physiological effects generally in response to stress situations, binding tissues specific intracellular receptors (*glucocorticoids receptors*, GR) and following the general steroids mechanism of genomic actions described above[49].

Like aldosterone, also cortisol is secreted with a circadian rhythm, following the ACTH secretion: its blood concentration is higher on wakening in the morning and it decreases throughout the day, reaching the lowest point in late evening (figure 2.7 adapted by *Williams Textbook of endocrinology*, 12th edition[48]). Individual ACTH and consequently cortisol secretion rhythm depends on subject's sleep-awake cycle, and also food intake is a stimulus for ACTH production. Important changes in the glucocorticoids daily secretion could be due to pathologies, such as the Cushing syndrome[48].

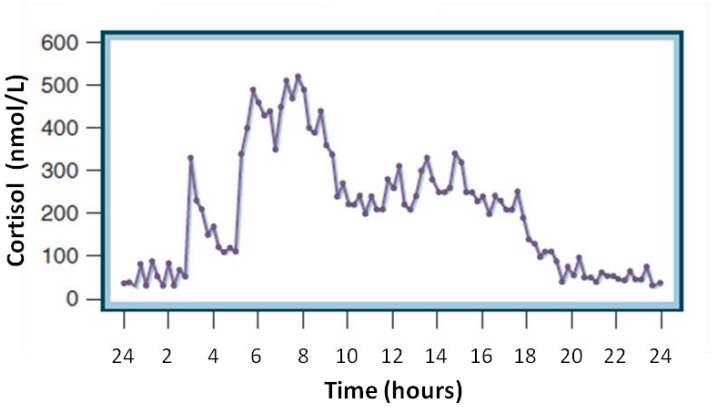


Figure 2.7: Cortisol circadian rhythm in healthy subjects.

Metabolic effects. Glucocorticoids have *catabolic* effect inducing mobilization of energy resources[48]. They stimulate *gluconeogenesis* in the liver, glucose de novo production, and inhibit *glycogenolysis*, the glycogen breakdown[51]; they inhibit glucose uptake in peripheral tissues, like the adipose tissue or the muscle, increasing the glucose blood concentration contrasting insulin action. Glucocorticoids can also stimulate proteins catabolism in extra-hepatic tissues, stimulate lipolysis in the adipose tissue, free fatty acids formation trough triglycerides hydrolysis and stimulate the adipocyte differentiation inducing adipogenesis, through the transcriptional activation of key genes[51].

Immune effects. Glucocorticoids have in general an anti-inflammatory effect[52], up-regulating the expression of anti-inflammatory molecules and down-regulating the expression of pro-inflammatory molecules, suppressing inflammatory responses and showing a regulatory role to prevent destructive reactions of the immune system, dangerous for the organism[52]. Glucocorticoids inhibitory effect on the immune system is exerted at different levels. In the blood, they can decrease the lymphocytes number, inducing their apoptosis; also the immunoglobulin production is decreased. They can inhibit cytokines synthesis inhibiting the *NF-kB* (*nuclear factor kappa-light-chain-enhancer of activated B cells*), an important transcription activator of cytokines genes[48]. Glucocorticoids can further reduce the local inflammatory response by preventing actions of histamine and down-regulate the prostaglandin synthesis.

Because of this inhibitory effect on the immune system, they are used for the treatment of pathological states caused by immune system hyper-activation, such as allergies and autoimmune diseases[53].

Central nervous system. It was shown that GRs are expressed in some key areas of rodents' brain such as hippocampus, hypothalamus, cortex and cerebellum[54]. Indeed glucocorticoids have various effects on the

mood in humans that can range from depression to euphoria[55]. It was also shown that high levels of glucocorticoids can cause neuronal death, especially in the hippocampus[56], impairing learning function.

Other effects. In general glucocorticoids increase the blood pressure, potentiating the action of vasoactive substances and acting on the kidneys and the vasculature. In the endocrine system, they suppress the secretion of various hormones, such as the *thyroid-stimulating hormone* (TSH), the *gonadotropin-releasing hormone* (GnRH), the FSH and the *luteinizing hormone* (LH)[48].

3. Sexual steroids

Sexual steroids are mainly produced by gonads, ovary, testicles and by the zona reticularis of the adrenal cortex. Similarly to glucocorticoids, the production of these steroids is regulated by the HPG axis through the production of the peptide hormone GnRH by neurons in the hypothalamus[48]. GnRH then acts on gonadotropin-producing cells of the anterior pituitary gland stimulating the production of LH and FSH, which can directly act on the gonads with a positive feedback mechanism inducing the sexual steroids production. The effect of sexual steroids can be mediated by intracellular and intranuclear receptors (*androgen receptors*, AR; *estrogen receptors*, ER; *progesterone receptors*, PR) inducing the slow genomic effect described before, or through plasma membrane G protein-coupled receptors inducing a fast non-genomic effect[48]. Sexual steroids are generally responsible for sexual characteristics development, but they are also involved in several other functions. These steroids can be divided in two main groups depending on their actions: *androgens*, sexual steroids class more abundant in males, and *estrogens*, more abundant in females.

Androgens. The primary steroid hormone of this class is the **testosterone**; its production occurs in Leydig cells of the testicles under the control of the LH, on which testosterone has a negative feedback, inhibiting the GnRH production in the hypothalamus[48]. Other androgens are the dihydrotestosterone (DHT), androstenedione and the DHEA. One of the primary functions of testosterone and other androgens is the development of the sexual male characteristics; during the embryogenesis, androgens production is essential for the normal male genital differentiation (primary sexual characteristics). The increase of circulating testosterone concentration during the puberty (figure 2.8 adapted by *Williams Textbook of endocrinology*, 12th edition[48]) is responsible for the normal development of secondary sexual characteristics, such as for instance the hair facial growth, and it is especially essential for the *spermatogenesis* and seminal fluid production. During the adult life, normal circulating testosterone levels are required to maintain many of the changes induced during the puberty[48]. Among other functions it was recently showed that androgens can also have a regulatory role decreasing fat deposits through the inhibition of adipocytes differentiation[57].

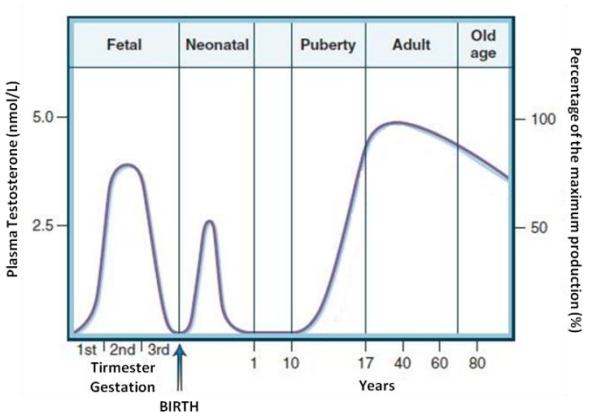


Figure 2.8: Testosterone levels during life.

Estrogens. In the steroidogenesis, these sexual steroids are directly derived from androgens androstenedione and testosterone. Estrogens are generally produced by the ovary, and like for androgens, also their production is under LH and FSH direct control and estrogens have a negative regulatory feedback on GnRH production. The primary hormones of this class are the **estradiol** (E_2), secreted by the granulosa cells of the dominant follicle, and **progesterone**, while other estrogens are estrone and estriol. The primary function of estrogens is the development of female sexual characteristics like endometrial and uterine growth, regulatory mechanism on the menstrual cycle and the oocytes maintaining in the ovary, all functions essential for the reproduction[48]. Indeed, in the luteal phase of the menstrual cycle estradiol and progesterone are necessary to prepare the endometrium for the implantation. Progesterone is the most important hormone during the pregnancy[48]: it allows the embryo implantation, it decreases the mother immune response against the fetus and it decreases contractility of the uterus muscles cells. Estradiol has also a role in male reproduction, preventing the sperm cells death[58]. Among other, non reproductive functions, estrogens have an important role in preserving appropriate bone metabolism[59]. Indeed during the menopause, when ovary production of estradiol and progesterone ceases, there is a higher risk of osteoporosis that leads to an increased risk of bone fracture.

Id. Steroid hormones related diseases

Since the great number of key functions that steroid hormones carry out in the body, a deregulation of their normal production or mechanism of action leads to a wide range of diseases.

1. Mineralocorticoids disorders

Two principal disorders involve mineralocorticoids production: *hyperaldosteronism* and *hypoaldosteronism*. Hyperaldosteronism is a pathological condition in which there is an increased aldosterone production in the adrenal cortex[60]. The first consequences of this over-production are *hypertension* and reduction of potassium blood levels, also called *hypokalemia*, due to the increased aldosterone action on the kidneys[60]. The hyperaldosteronism can be primary, direct consequence of hyper-production by adrenal glands, or secondary, indirect consequence of other pathological conditions. The primary is usually caused by an adrenal adenoma, called *Conn's syndrome*, or by an *idiopathic adrenal hyperplasia*[61] (75% of clinical cases). The discrimination between these two pathological conditions is essential because the treatment for each is different: for Conn's syndrome the most effective therapy is the surgery intervention, while in the case of idiopathic adrenal hyperplasia the treatment is a medical therapy with aldosterone antagonists[62]. The secondary hyperaldosteronism is due mainly to over-activation of the renin-angiotensin system, as for instance in the presence of a renin secreting tumor, which leads to an increased aldosterone production. Recent evidences have shown an increased function of the renin-angiotensin-aldosterone system in obesity[63]. An increased aldosterone production was found in obese patients[64] and it may contribute to obesity-related hypertension and the increased risk of cardiovascular disease. Further evidences highlighted a possible role of adipokines, cytokines-like molecules produced by the adipose tissue, as stimulus for the increase of aldosterone production[65].

Hypoaldosteronism, on the other hand, is the pathological condition in which aldosterone production is decreased[60]. The causes of primary hypoaldosteronism can be a general adrenal insufficiency or genetic causes like several enzymatic disorders resulting from mutations in genes coding for enzymes responsible for the steroidogenesis. In particular for aldosterone the most important enzyme lacking is the *aldosterone synthase* (or *18 hydroxylase*) that catalyses the conversion of corticosterone in aldosterone[66]. The hypoaldosteronism causes hypotension due to hypovolemia and hyperkalemia, the increase of blood potassium.

2. Glucocorticoids disorders

One of the most diffused disease involving glucocorticoids is the *Cushing's syndrome* that can be defined as the pathological state due to prolonged exposure to elevated levels of circulating glucocorticoids[67]. It can be caused by endogenous increased production of glucocorticoids or it can be *iatrogenic* when it is due to long term therapies with glucocorticoids drugs. The most diffused form of Cushing's syndrome with an endogenous cause has a pituitary origin, often the result of the presence of a benign pituitary adenoma secreting ACTH in excess[67], which stimulates continually glucocorticoids production by the adrenal gland. Another endogenous cause can be an adrenal adenoma secreting glucocorticoids or an ectopic production of ACTH, for instance in presence of lung cancer[68]. The symptoms are mostly gonadal dysfunctions with menstrual irregularity in women, depression and lethargy, and a marked obesity with diabetes in most of the cases[69]. Accurate cortisol and ACTH levels determination is indispensable to identify this syndrome; especially the deregulation of their circadian rhythms can be an important clue. Also some asymptomatic tumors, called *incidentalomas* because detected by coincidence, can secrete glucocorticoids in excess (30% of adrenal incidentalomas)[70] giving rise to a *subclinical Cushing's syndrome*, defined as a glucocorticoids abnormal production without specific Cushing's syndrome symptoms. Another disease involving the glucocorticoids production is the *Addison's disease*, an autoimmune pathology characterized by adrenocortical insufficiency and therefore glucocorticoids (and mineralocorticoids in some cases) deficiency[71]. The primary adrenocortical insufficiency can be caused by different metastatic tumors and ACTH resistance syndrome[48]. Interestingly, Cushing's syndrome and Addison's disease are characterized by opposite modification of the metabolic phenotype: while marked obesity is a primary trait of Cushing disease and therefore of the glucocorticoids excess, Addison's disease is characterized by weight loss and hypoglycemia; both these conditions evidenced the critical involvement of glucocorticoids in energy balance[72]. The secondary adrenocortical insufficiency, on the other hand, can be due to hypopituitarism or sudden interruption of glucocorticoids therapies (iatrogen).

The *congenital adrenal hyperplasia* (CAH) is a group of autosomal recessive disorders characterized by mutations of the genes coding for enzymes involved in the steroidogenesis[73]. In almost every case of CAH there is a defective cortisol production[73] and, depending on the steroidogenesis pathway involved, there could be also a mineralocorticoids or androgens subnormal production. Usually, there is a hyper-production of the steroid hormone upstream the defective enzyme and a deficit of all the steroid hormones downstream. The most diffused enzyme lacking is the CYP21 also known as 21 hydroxylase (95% of all the CAH[74]) located in the SER of adrenal cells, that catalyzes the conversion of progesterone in DOC in the mineralocorticoids pathway and the conversion of 17OH-progesterone in 11-deoxycortisol in the glucocorticoids pathway (figure 2.9). This results in a decreased aldosterone and cortisol production that leads to hyperplasia of the adrenal cortex and elevated ACTH levels, due to the absence of cortisol negative

feedback. There could be also an increased androgens production, because the excess of 17OH-progesterone that cannot be converted in the glucocorticoids pathway is converted to androstenedione[74] (figure 2.9). The principal signs for a 21 hydroxylase deficiency diagnosis in newborns are salt wasting, hypotension, hypoglycemia and genital ambiguity in females due to the androgens excess. In every case of CAH, a correct steroids circulating profile determination is essential to determinate the diagnosis and the lacking enzyme[73].

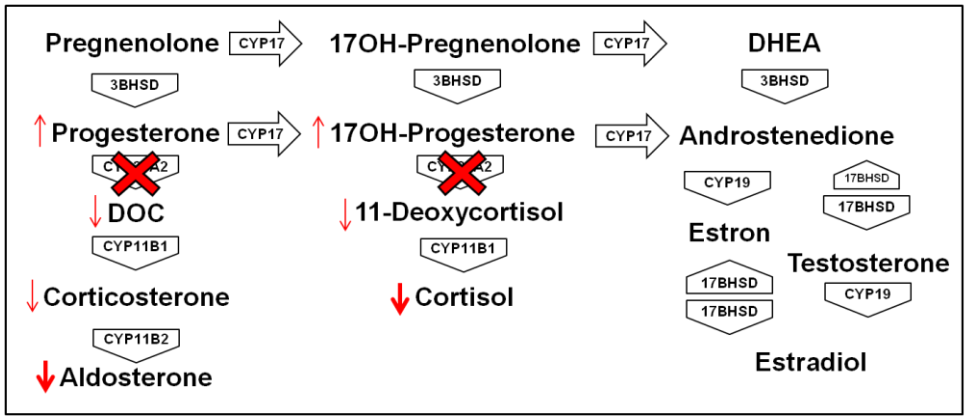


Figure 2.9: 21-hydroxylase deficiency: effects on steroidogenesis.

3. Sexual steroids disorders

Among pathological conditions concerning the sexual steroids, the *hypogonadism* can be defined as the clinical syndrome that results from a decreased functional activity of the gonads and therefore a reduced sex steroids biosynthesis[48]. More specifically, *hypoandrogenism* in men is the clinical syndrome that results in a decreased testosterone, and consequently sperm production by testicles[48]. The consequences of the androgen deficiency are different depending on the onset age and the severity of the deficit. In fetal androgens deficiency, usually caused by congenital lack of testosterone biosynthesis enzymes, the phenotype may range from a normal female to a male with ambiguous genitalia, depending on the degree of androgens production. During the pre-pubertal age, testosterone levels rise allowing secondary sexual characteristics development and during the adulthood is necessary to maintain all the sexual functions; in presence of androgens deficit these processes are impaired, resulting in patients with impotence, infertility,

gynecomastia and poor muscle mass strength[48]. Non congenital hypoandrogenism may have different causes like tumors, autoimmunity, drugs and many others[48]. Furthermore, a relationship between obesity and decreased androgens circulating levels has been extensively documented[75] but molecular mechanisms underlying this decreased production are still not well defined.

Hypoestrogenism is the clinical syndrome consisting in a decreased estradiol production in the ovary. It is physiologic in post-menopausal age, when estrogens production falls, but it can be also associated to drugs treatments against endometriosis, like GnRH antagonists[76]; also in this case the consequences are similar to normal menopause symptoms, like for instance hot flashes and lower libido. The *hypergonadism* is the opposite condition, in which there is a hyper-functionality of the gonads and therefore increased levels of circulating sexual steroids[48]. Commonly, the *hyperandrogenism* in women is usually one of the primary symptom of the *polycystic ovary syndrome* (PCOS) frequent in post-pubertal age and associated with irregularity of the menstrual cycle and, as long term consequences, dyslipidemia and insulin resistance[77], whereas the hyperestrogenism in most of the cases is due to secreting ovarian tumors[78]. Furthermore in both sexes, dysfunction of the enzyme *aromatase* (CYP19) can cause abnormalities in sexual hormones circulating levels: this enzyme catalyzes the conversion of testosterone in estradiol and the conversion of androstenedione in estrone in many tissues. As a consequence, the *aromatase excess syndrome* (AEXS) causes an excess of estrogens resulting in gynecomastia and adult hypogonadism in males and menstrual irregularity and enlarged uterus in females[79] , while aromatase deficiency can lead to virilization in females and osteopenia or osteoporosis in men due to low levels of estrogens[80].

Ie. Glucocorticoids, androgens and obesity

In addition to all the pathologies described so far, also obesity can alter steroid hormones production. Visceral obesity is associated, among other alterations, with a hyper-activation of the HPA axis, leading to an altered glucocorticoids action, and a sex specific impairment of the HPG axis[10].

The cause of the HPA axis hyper-activation is still unclear; a hypothesis is represented by the fact that obesity can be assumed as a chronic stress condition, leading to a chronic activation of this neuro-endocrine axis[81]. On the other hand, this hyper-activation could represent also a compensatory mechanism for an increased clearance of cortisol due to an altered function of the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11βHSD) in the adipose tissue[75].

However, the direct relationship between circulating cortisol levels and visceral obesity can be explained by the human pathophysiological models of the Cushing's syndrome and the Addison's disease[72]. As explained

before, Cushing's syndrome is characterized by increased cortisol levels and the visceral obesity is a distinctive tract of the Cushing phenotype, whereas the Addison's disease presents decreased cortisol production due to adrenal insufficiency and anorexia and weight loss are characteristics of this pathology[72].

Although increased circulating concentrations of cortisol have been found in obese patients[82-84], the fact that the hyper-cortisolism is a direct consequence of increased visceral fat is not as evident. In some studies it has been found a direct correlation between cortisol circulating levels and waist circumference[85-86], whereas in others this association was lacking[83]. Further knowledge is therefore required to elucidate this intricate link between the hyper-activation of the HPA and obesity.

The impairment of the HPG axis is different according to the gender[10]. In men, increase of body weight and visceral adiposity are associated with decreased levels of testosterone, reduced SHBG and increase in estrogens levels, impairing the sexual functions[75]. The cause-effect relationship is still not clear; obesity and testosterone low levels seem to have a bidirectional link: low testosterone levels predict development of visceral obesity while high BMI and central adiposity lead to decreased circulating testosterone[87].

In general, obesity is one of the main chronic pathologies associated with testosterone deficit in men[88], and the molecular reasons underlying this mechanism are still poorly understood[89]. Androgens levels decrease proportionally to the increase of BMI[90], suggesting that this decline is a direct consequence of increased visceral fat accumulation. Interestingly, a direct inverse correlation was found between circulating leptin and circulating testosterone in men with different obesity degree[90]. Since obese patients have higher levels of circulating leptin[8], it is possible that this leptin excess can contribute to the reduction of testosterone, also because leptin receptors are present in Leydig cells, responsible of testosterone production[90]. Moreover, several studies found normal levels of gonadotropin in obese patients, suggesting that the inhibition of testosterone production is not due to central mechanisms[90]. Further studies are however required to confirm these hypotheses and to elucidate this obesity-related impairments.

In contrast, in women obesity is associated with increased secretion rates of testosterone and DHT[2]. Moreover, in obese women SHBG concentration is inversely correlated with the increase of body weight[91]; this determines an increase of free testosterone and DHT available in tissues[10]. However, only few studies were performed so far to determine the relationship between androgens increase and obesity in women; furthermore, results in the literature are not consistent[92].

II. Steroid Hormones measurement: state of the art

Since an accurate measurement of circulating steroid hormones is essential to correctly address the diagnoses of each pathological condition described in the former section, the development of suitable and reliable quantitative methods for these molecules has been since long time the aim of many analytical chemistry researchers. Steroids represent a wide class of hormones in which molecules are structurally very similar to each other and furthermore the most part of these analytes circulate in the blood at very low concentration; for this reason the analytical method of choice has to be sensitive, specific, precise and accurate.

So far, there are three main analytical methods used in the research and clinical practice to quantify steroid hormones: RIA, GC/MS and LC-MS/MS. Here I will review one by one these techniques, in order to evaluate the best to develop a quantification method for steroid hormones.

IIa. Radioimmunoassay and Immunoassay

Radioimmunoassay is an analytical quantification method developed by Rosalyn Sussman Yalow and Solomon Aaron Berson in 1960 for the first time to measure insulin in human plasma[40]. For this discovery they received the Nobel Prize in medicine in 1977. In the 1969 the first RIA for steroid hormone was developed by Abraham, measuring estradiol in serum[13]. Since its discovery, RIA has been the most used technique for hormones circulating concentration measurements; indeed, this technique joints the specificity of an immunologic reaction (antigen-antibody complex) with sensitivity of a radiochemistry method and at the moment is the most used technique for steroids quantification in the clinical routine. The two key components of this assay are: a known amount of mixture containing the radioactive antigen (usually labeled with ^{125}I or ^{131}I that can be easily introduced into protein tyrosine residues) and a known amount of highly specific antibody to bind the antigen. The radiolabeled antigen and the correspondent antibody are then mixed and the formation of the complex antigen-antibody occurs. An amount of this mixture is then added to pre-purified blood samples: the “cold” (unlabeled) antigen from the serum competes with the radiolabeled one for the binding site of the antibody (figure 2.10). The bound radiolabeled antigen is then separated from the unbound one adding a secondary antibody and the radioactivity of the latter is measured. By building a calibration binding curve with pure standard analytes, it is possible to calculate the concentration of analytes in the serum sample. The primary RIA advantage is the sensibility due to the radioactivity. The limits of this technique are the cost of the kits and of the radioactive waste disposal and especially the specificity lack: commercially available antibody can present *cross-reactivity*, binding also molecules structurally similar to the analyte, leading to an overestimation of

the analyte concentration[12]. In particular, the specificity lack is a dramatic issue for molecules with a very low concentration, such as testosterone in women. In 2003, Herold and Fitzgerald in a Clinical Chemistry editorial clearly titled "*Immunoassays for testosterone in women: better than a guess?*"[41] affirming that concentrations obtained using some commercially available RIA kits measuring testosterone in women were always inaccurate missing a lot the target values. Also estrogens levels in post-menopausal women, an important clue to predict the breast cancer risk, are currently determined using RIA, achieving unreliable results in most of the cases, due to their very low concentration. In a review of 2010, the authors explained that RIA results for these analytes presented inter-individual differences in specificity causing significant problems when used for epidemiologic studies of breast cancer[93]. Furthermore, another limit of this analytical technique is the impossibility for the commercially available kits to measure different analytes simultaneously: the classical RIA kit approach is to measure each steroid using individual aliquot of serum analyzed with an assay made for a single analyte[42]. Therefore, to evaluate a whole steroid pattern for a complex endocrine disease is necessary to use some different kits, making the procedure costly and time consuming. To overcome the RIA time-consuming issue in the clinical routine, laboratories are currently using *immunometric platforms*, a completely automatized system that requires a small sample volume (approximately 0.1-0.2 ml) to measure multiple steroids from a single serum aliquot, using *chemiluminescence* or *electrochemiluminescence* instead of the radioactivity. However, these platforms also have serious advantages due to the specificity lack: the sample purification is not performed leading sometimes to an overestimation of the analyte concentration when the antibody binds structurally similar molecules and to an underestimation when matrix interferents compete with the analyte binding the antibody.

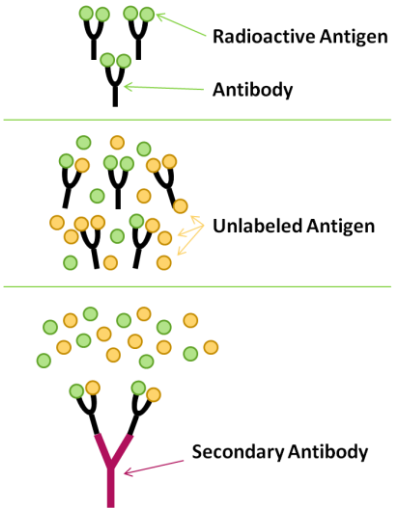


Figure 2.10: The Radioimmunoassay.

IIb. Gas chromatography - mass spectrometry

GC/MS is currently one of the most reliable quantification techniques for circulating steroid hormones and as such it is considered the reference method also called *gold standard*. It was used during 60s to elucidate the structures and the biosynthesis pathway of this class of hormones[94]. The GC/MS offers a high comparable level of sensitivity and specificity to LC/MS, basing the analysis on the same technical principles but with different features. In brief, the mixture of analytes is separated on a silica capillary column with a very small diameter (< 0.50 mm) and a length not lower than 10 m containing the stationary phase; the mobile phase is an inert gas that pass through the column dragging out the analytes at different times depending on the physical characteristics of the analytes and on the chemical nature of the stationary and mobile phase of choice, following the traditional chromatography principles. Afterwards, the sample is introduced in the mass spectrometry and then ionized, allowing the ions corresponding to the molecules of interest to reach the detector where they can be identified and quantified. The most important difference between the GC/MS and LC/MS, representing also the limit of this technique, is that the chemical nature of the analyte can be a limit to analyze it, since only volatile and thermo-stable compounds are completely suitable for this kind of chromatography. Most analytes, including also steroids, are not intrinsically volatile and as such not analyzable with GC/MS. In these cases, it is used the *derivatization procedure*[42]: analytes moieties are chemically modified to make compounds more volatile and thermo-stable. This procedure has some disadvantages: the pre-analytical sample preparation is more time consuming and hard working[42] making the introduction of GC/MS complicated in clinical routine laboratories. Furthermore, some different moieties added to analytes by the derivatization procedure could bind the analyte at different sites, generating from the same molecule compounds of different molecular weight complicating analyte identification. The advantage of the GC/MS compared to LC/MS is the more elevated resolution of the peaks also for molecules with minimal structure differences and the more accurate qualitative analysis: some kind of derivatizations, such as the *methyloxime-trimethylsilyl ether* (MO-TMS), allow a rapid structural characterization of the molecule, easily predicting the number of C=O and C-OH groups[94]. Particularly interesting is the opinion of same authors affirming that in the steroid hormones analysis field LC/MS and GC/MS can become complementary technique rather than competitive, since LC/MS is more suitable for steroids clinical routine quantification, while GC/MS is more useful for the structural analysis in the research field[94].

IIc. Liquid chromatography tandem mass spectrometry

Since all the analytical methods used so far for steroid hormones quantification have some significant limits, in the past decade LC-MS/MS development has been a turning point in the field of steroid hormones

quantification[95]. This technique represents the best compromise between RIA applicability and GC/MS advanced reliability, having high sensitivity and specificity parameters values and avoiding the time consuming derivatization procedure, since there are not chemical limits for molecules analysis[94]. The LC-MS/MS is capable to quantify different steroid hormones in a single chromatographic run from a single serum aliquot, introducing the revolutionary ability to achieve high throughputs with the minimum time-consuming sample preparation, consisting in simple and fast sample purification without the derivatization step[13]. In the past decade, a large number of steroids quantification methods were developed, becoming always more sensitive and fast, satisfying the needs of clinical laboratories[43-44]. However, the limits for a wide use of this technique in the clinical practice still are the great initial investment for the equipment and requirement of staff with specialized expertise.

IId. Analytical techniques comparison

As explained in the previous paragraphs, the three analytical techniques used so far for steroids quantification RIA or immunoassay, GC/MS and LC-MS/MS have different features.

The requirement of specific chemical characteristics of analytes is one of the main disadvantages of GC/MS: as told before, only volatile molecules can be analyzed with this technique and when molecules do not have this propriety, such as steroids, a derivatization step has to be performed to chemically modify analyte moieties. On the contrary, LC-MS/MS has no limitations about chemical nature of the analytes of interest because of the versatility of the liquid chromatography. Furthermore, LC-MS/MS can be easily automatized, while GC/MS, requiring the derivatization step, cannot. While the sensitivity of all the three discussed methods is excellent, specificity represent the most important limit of RIA, critically linked to the specificity of the antibody commercially available. Mass spectrometry techniques offer a great specificity, increased with the MS/MS analysis. Furthermore, since the possibility to develop multi-analytical methods, the daily potential throughput is significantly higher with mass spectrometry techniques; certainly, LC-MS/MS offers shorter analysis time compared to GC/MS: after a simple and fast purification the sample can be directly injected into the system without additional steps. Additionally, RIA cost per sample is definitely higher, whereas for LC-MS/MS the cost per sample is related to common solvents and consumables, once afforded the initial instrument cost. Different technical features of RIA, GC/MS and LC-MS/MS are summarized in table 2.1.

Giving the high sensitivity and specificity, wide applicability, reduced time-consuming, high productivity and reduced cost per sample, we decided to employ LC-MS/MS to develop a new fast, multi-analytic, sensitive and specific method for the quantification of steroid hormones in blood.

	Task	RIA	GC/MS	LC-MS/MS
Applicability	**Molecular weight limit**	No limit	Linked to instrumental features	Linked to instrumental features
	Chemical analyte nature	No limit	Analytes volatile and thermostable	No limit
Pre-analytic	**Sample preparation**	Sample purification	Sample purification	Sample purification
	Derivatization	Not needed	Necessary	Generally not needed
	Use of radioactivity	Yes	No	No
	Automatized?	Yes	No	Yes
Analytic	**Method development**	Industrial (kits)	"Home-made" with technical experience	"Home-made" with technical experience
	Sensitivity	Generally good	Generally good	Generally good
	Specificity	Limited	Generally good	Generally good
	Speed of the analysis	Long	Medium	Short
Post-analytic	**Maintenance**	Quite easy	Quite easy with technical experience	Quite easy with technical experience
	Daily throughput	100 measurements of the same analyte	50 measurements multi-analyte	50 measurements multi-analyte
	Cost per sample	High	Low	Low
	Cost of the equipment	High (detector) Medium (kits)	High	High

Table 2.1 : Different features of RIA, GC/MS and LC-MS/MS

III. Aim of the project

Steroid hormones are a wide class of molecules carrying out many essential functions in human physiology. An impairment of their circulating levels, given by alterations of their synthesis or mechanisms of action can lead to a wide class of severe pathologies. We have been interested in the quantification of these molecules, developing an analytical method and establishing new reference intervals in normal and obese subjects. A precise and accurate quantification of these circulating molecules is indispensable to diagnose steroid-related pathologies, such as enzymatic deficit or adrenal insufficiency, and to monitor patients follow-up.

So far, quantification methods for these molecules were carried out with RIA, immunoassays or GC/MS. Making a careful comparison of these two techniques with LC-MS/MS we decided to use the latter to develop a new quantification method for steroid hormones. Indeed, as told in the previous section, LC-MS/MS represents the best compromise between RIA applicability and GC/MS specificity. The MS/MS analysis provides a superior specificity level. Therefore we developed a fast, sensitive and specific method for nine steroid hormones, chosen for their biological relevance, but also for common chemical characteristics that allowed us to develop a multi-analytic method. Separation and quantification of cortisol, corticosterone, 11-deoxycortisol, androstenedione, testosterone, DOC, 17OH-progesterone, DHEA and progesterone was achieved in 21 minutes. The method has been then validated following the FDA guidelines for the validation of bioanalytic methods[45] evaluating sensitivity and linearity, specificity, accuracy and precision, matrix effect and consequent analytes recovery.

Once obtained the values from the validation, we decided to carry out a comparative study with the immunoassay approach, currently used at the Laboratorio Centralizzato of the Policlinico Sant'Orsola in Bologna to measure steroid hormones in the clinical routine. A comparison study was carried out for six out of nine analytes, since corticosterone, 11-deoxycortisol and DOC at the time of the study were not measured at the Laboratorio Centralizzato. Circulating values of cortisol (n = 159), testosterone (n = 162) progesterone (n = 85), androstenedione (n = 137), DHEA (n = 143) and 17OH-progesterone (n = 99) were measured by LC-MS/MS and obtained results were compared with values obtained by immunoassays.

Results of our comparative study confirmed the already known limits of the immunoassay, especially regarding analytes circulating at low concentrations, such as testosterone in women or progesterone < 1 ng/ml. Giving that, we decided to perform a screening on a healthy population, to redefine steroids reference values in normal population. Currently, reference intervals used in the clinical practice were made with RIA in 50s; because of the well-identified limits of these immunometric techniques, it is urgent a review of these reference values. We recruited 179 healthy males and 177 healthy females grouped by age, among the blood donors in the Centro Trasfusionale of the Policlinico Sant'Orsola. The redefinition was

made for eight out of nine steroid hormones, since in the majority of the population DOC circulating values were below the limit of quantification of our method.

Once established circulating ranges in the healthy population, we decided to measure circulating steroids levels in an overweight population (n= 88, BMI: 25.1 – 30.0 kg/m^2) and in an obese population (n=93, BMI > 30 kg/m^2). A group of healthy control subjects, matched with obese and overweight subjects by age and anthropometric characteristics, was extrapolated from the healthy population recruited for the steroids reference interval study.

The relationship between obesity and steroid hormones is long known. Obesity leads to impairment of both the HPA axis and the HPG axis, causing altered functionalities and altered circulating concentrations of steroids, especially regarding glucocorticoids and androgens[14]. The direct link between this deregulation and the obesity condition is still poorly understood. Since among molecules included in our method are present some of the principal glucocorticoids (cortisol and 11-deoxycortisol) and androgens (testosterone, DHEA and androstenedione) and also an intermediate of both glucocorticoids and androgens synthesis (17OHprogesterone), we decided to use this method to find differences in steroid profiles among subjects with different BMI. Our findings will be useful to better understand the changes induced by obesity in the HPA axis and HPG axis, and the relationship between circulating steroids impairment and the development of obesity co-morbidities.

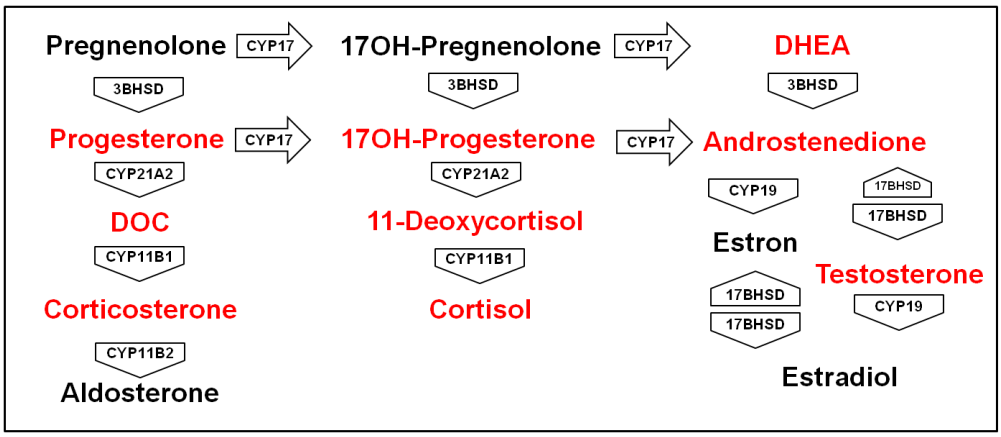


Figure 2.11: Steroid hormones included in the quantification method (in red).

IV. Materials and methods

IVa. Chemicals

Pure standard of cortisol, corticosterone, 11-deoxycortisol, androstenedione, DOC, testosterone, 17OH-progesterone, DHEA, progesterone, 21-deoxycortisol, epitestosterone, DHEA-sulphate (DHEA-S) and cortisone were from Steraloids, Newport, RI. Pure standard of deuterated compounds such as d4-cortisol, d8-corticosterone, d2-11deoxycortisol, d5-testosterone, d8-17OHprogesterone, d2-DHEA and d9-progesterone were from CDN Isotopes, Pointe Claire, Canada while C2-testoterone was from Cambridge Isotopes Laboratories, Andover, MA. Bovine serum albumin (BSA) and pure standard of prednisone and prednisolone were from Sigma-Aldrich, St. Louis, MO; pure standards of betamethasone, dexamethasone 21phosphate disodium salt, methylprednisolone acetate and triamcinolone acetonide were respectively from Defiante Farmaceutica, Madeira, Portugal; Visufarma, Rome, Italy; Pfizer, New York City, NY; Bristol-Myers Squibb, New York City, NY. Gradient grade methanol and zinc sulphate hepta-hydrated were purchased from Merck, Darmstadt, Germany; ultra-pure water was produced by MilliQ Gradient A10 system (Millipore, Volketswill, Switzerland). Steroid-free serum was from MP Biomedicals, Solon, OH. The solid phase extraction (SPE) cartridges were IST Isolute C18 100mg, 1cm^3 from Biotage, Uppsala, Sweden. Reference material was from the *Reference Institute for Bioanalytics*, Bonn, Germany.

IVb. Steroids immunometric measurements

For the method comparison study, we evaluated immunometric methods routinely used in the clinical routine by the Laboratorio Centralizzato of the Policlinico Sant'Orsola Malpighi in Bologna. Cortisol, testosterone and progesterone were measured by electro-chemiluminescence immunoassay on the Modular Analytics ElecsysE170 by Roche Diagnostics (Mannheim, Germany); androstenedione by the solid-phase, competitive chemiluminescent enzyme immunoassay Immulite2000; DHEA by DSL-9000 RIA and 17OHprogesterone by 17OHP Bridge RIA. 11deoxycortisol, corticosterone and DOC were excluded from the comparative study, since there are no immunoassays performed for this analytes in the Laboratorio Centralizzato.

IVc. Biochemical and blood parameters measurements

Circulating triglycerides (CV intra-assay: <1.5%; CV inter-assay: 1.8%), total cholesterol (CV intra-assay: 1.0%; CV inter-assay: 2.7%), cholesterol high density lipoprotein (HDL) (CV intra-assay: 0.95%; CV inter-assay: 1.3%), insulin (CV intra-assay: 1.5%; CV inter-assay: 4.9%) and uric acid (CV intra-assay: 0.5%; CV inter-assay: 0.7%) measurements were performed by the Laboratorio Centralizzato at the Policlinico Sant'Orsola Malpighi in Bologna. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were determined using a sphygmomanometer Gamma G5 (Heine, Herrsching, Germany); glycemia was measured with a glucose meter Breeze 2 Bayer (Leverkusen, Germany).

IVd. Subjects and statistics

Patients recruitment has been made in different hospital facilities; indeed, different groups of subjects participated to each study. For the comparison study, we analyzed de-identified sera from the Laboratorio Centralizzato. For the steroids reference intervals redefinition, healthy subject were recruited among blood donors. Some of these healthy subjects were included in the control normoweight group then compared with overweight and obese volunteers in the last study.

i. Samples for quantification methods comparison study

De-identified serum samples were collected for the method comparison study among sera from the Laboratorio Centralizzato of the Policlinico S.Orsola-Malpighi. The LC-MS/MS method was compared with immunoassay measurements for analytes cortisol (n = 159), testosterone (n = 162) progesterone (n = 85), androstenedione (n = 137), DHEA (n = 143) and 17OHprogesterone (n = 99). All the results below the LOQ of both methods were excluded. Non-normally distributed variables were compared by the *Mann–Whitney test* and all data are expressed as median and interquartile range (IQR). To assess the imprecision of both the quantification methods was used the *Deming Regression*[96]. *Bland Altman* plots[97] were used for the methods agreement estimation, representing the percentage difference between the methods against the mean.

ii. Subjects recruitment for estimation of steroids reference intervals

The subjects were recruited among healthy blood donors at the Centro Trasfusionale of the Policlinico Sant'Orsola in Bologna and among healthy volunteers at the local health service of the town of Massa

Lombarda for the project *Regione Emilia Romagna*, after their informed consent. 179 adult males and 177 adult females were selected and grouped by age; for pre-menopausal females there are two subgroups depending on the menstrual cycle phase (follicular, day 1 - 11 or luteal). Subjects were normoweight (BMI 18 – 25) with a waist circumference lower than 88 cm for women and lower than 102 cm for males and free from any kind of drugs during the period of the study. All the inclusion and exclusion criteria are summarized in table 2.2. The blood withdrawal was performed between 8:00 and 10:00 a.m. after ten minutes of saline solution infusion. Data analysis was performed on MedCalc v9.3.7.0 (Mariakerke, Belgium).

INCLUSION CRITERIA	EXCLUSION CRITERIA
Age: 18-90 years	Any kind of drug in the last 4 weeks
BMI: 18-25 kg/m^2	Drug or alcohol addiction
Body weight stability in the last 3 months	Endocrine diseases
Normal sexual development	Kidney or liver diseases
	Heritable disorders
	Current or previous cancer
	Current or previous autoimmune diseases
	Current or previous cardiovascular diseases
	Current or previous neurological diseases
	Current or previous psychiatric diseases
	Chronic intestinal diseases
	Allergy
	Sleeping disorders

Table 2.2: Inclusion and exclusion criteria for the redefinition of steroids reference intervals.

iii. Obese subjects recruitment for assessment of steroids profiles

Among the healthy volunteers recruited at the local health service of the town of Massa Lombarda for the project Regione Emilia Romagna, 34 males and 58 females obese (BMI>30.1) were selected. They were not diabetic and they were not taking any kind of drug at the moment of the study. This obese population was compared to 34 males and 54 females overweight (BMI: 25.1 – 30.0) and to 34 males and 59 females normoweight (BMI: 18.0 – 25.0) of the same age. All the characteristics of the three populations were compared using the *Kruskall Wallis* test. Significant differences in circulating steroids were then analyzed with *Dunn's multiple comparison test*.

V. Results

The quantification method was developed by first identifying the best mass spectrometry and chromatography condition to obtain the best signal from all the nine analytes: cortisol, corticosterone, 11-deoxycortisol, androstenedione, DOC, testosterone, DHEA, 17OH-progesterone and progesterone. The extraction was optimized allowing the best recovery percentage and sensitivity. First, we validated the quantification method following the FDA guidelines[45] by calculating sensitivity and linearity, specificity, accuracy and precision, matrix effect and recovery percentage.

The quantification method was then used to measure steroid hormones in approximately 300 de-identified sera to carry out a comparative study with immunometric techniques currently used in the clinical routine to quantify these hormones. After this, we used our method to redefine circulating ranges of these nine steroids in healthy subjects, since the reference intervals used so far are at this point obsolete.

Once obtained circulating ranges in healthy subjects, we measured steroids in an overweight and an obese population, to test the differences induced by a different BMI class or obesity condition.

Va. Quantification method development

1a. Mass spectrometry parameters assessment

i. Mass spectrometer: API 4000QTrap instrument

The mass spectrometer used for this part of the project is a triple quadrupole API 4000QTrap AB Sciex (Toronto, Canada). This instrument is equipped with an interchangeable ionization source that can operate in two different ionization modes: ESI (*TurboIonSpray*^{TM}) and APCI (*Heated Nebulizer*^{TM}). Ions generated in the source at atmospheric pressure are then introduced in the quadrupole region through an orifice located in the middle of a metallic plate, called *curtain plate*. The *curtain gas*, a nitrogen stream with an opposite direction respect to the ions flow, allows only charged molecules to go through the orifice, removing interference molecules while the *exhaust gas*, connected to a waste flask, eliminates liquid excess in the source. Both these gas streams protect the high vacuum spectrometer region. After the curtain plate there is a ceramic plate, called *orefice plate*, with a further smaller orifice allowing the ions entrance in the quadrupole region. Ions are here accelerated by a voltage, called *declustering potential* (DP), applied between the orifice plate and a lens called *skimmer*: high DP values correspond to ions that

need high energy to detach themselves from solvent and water molecules. The *entrance potential* (EP) then drives the ion beam through the Q0, an accessory quadrupole used to stabilize the ions trajectories. The interface is summarized in figure 2.12. The Q0 is a region in which there is a medium vacuum, $8x10^{-3}$ torr, while the operative three quadrupoles are in a region with high vacuum, $3x\ 10^{-5}$ torr. Before each quadrupole there is a *quadrupolar lens* and a pre-filter called *stubbies* (ST), essential to preserve the voltage and to focus the ion beam.

In the second quadrupole-collision chamber occurs the ions fragmentation, also called *collision activated dissociation* (CAD); this "special" quadrupole is therefore equipped with an inert gas flow, such as nitrogen, also called *CAD gas* essential for the fragmentation. The API 4000QTrap collision chamber, the LINAC™ has particular inclined rods, which allow acceleration of the ion beam. In this way, the collision chamber can be emptied from the generated fragments in 5msec and be rapidly ready for the following analysis, avoiding the overlap of different parent ions fragments. It is possible to set two compound dependent parameters for the Q2: the *collision energy* (CE) is the voltage between Q0 and Q2 that determines the kinetic energy used by the ions to collide with nitrogen molecules generating fragments and the *cell exit potential* (CXP), the voltage between Q2 and Q3, which determines the speed of the ions entering in the Q3.

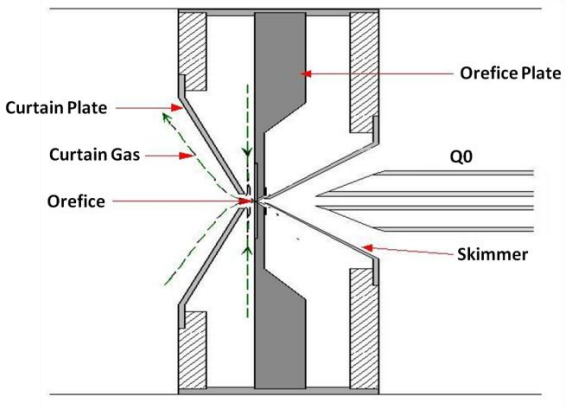


Figure 2.12: API 4000QTrap interface.

ii. Optimization of syringe pump parameters

For each analyte and deuterated internal standards (ISTD) two specific transitions were characterized, a "quantifier" for the quantitative measurement and a "qualifier" to further confirm the identity of the analyte, using the MRM mode. The electric parameters "compound dependent" were assessed in this phase, infusing standard solutions of each analyte directly in the source at concentrations ranging from 100ng/ml to 10ug/ml through an infusion pump (Harvard Apparatus, Holliston, MA) and a Hamilton 1001 TLL 1ml syringe (Supelco, Bellefonte USA) providing a constant flow of 10ul/min.

There are different stages of the syringe pump analysis:

Q1 scan: identification of the *parent ion* observing all the ions included in a certain range of m/z.

Q1 multiple ions: increasing values of DP and EP were applied to the analyte m/z value previously found in Q1; for both the parameters, the value giving the higher analyte intensity was chosen.

Q2 product ions: The parent ion was fragmented in the collision chamber. The whole fragmentation mass spectrum was evaluated and the two most abundant and characteristic fragments were chosen ("quantifier" and "qualifier"), to obtain the higher sensitivity and specificity. Afterwards, increasing values of CE and CXP were applied and the values giving the highest fragment intensity were chosen.

At the end, optimized m/z, DP, EP, CE and CXP of every analyte and ISTD were included in the final MRM experiment used to set the chromatographic parameters. All the parameters are summarized in the table 2.3.

Analyte	MW	Tr	Transition	m/z Q1	m/z Q3	DP	CE	CXP	IR
Cortisol	362.46	7.4	Quantifier	363.2	121.2	60	45	3	4.3
			Qualifier	363.2	267.4	60	35	5	
d4-cortisol	366.46	7.4	IS	367.3	97.1	50	45	3	
Corticosterone	346.46	9.5	Quantifier	347.1	121.0	76	45	9	2.1
			Qualifier	347.1	97.1	76	45	5	
d8-corticosterone	354.46	9.4	ISTD	355.4	125.4	88	45	5	
11Deoxycortisol	346.46	9.9	Quantifier	347.2	109.1	82	45	5	1.1
			Qualifier	347.2	97.0	82	45	5	
d2-11 Deoxycortisol	348.46	9.9	ISTD	349.4	97.1	75	45	3	
Androstenedione	286.41	11.3	Quantifier	287.4	97.0	78	30	3	1.4
			Qualifier	287.4	109.0	78	40	5	
DOC	330.50	11.6	Quantifier	331.4	109.1	80	40	4	1.1
			Qualifier	331.4	97.0	80	30	4	
Testosterone	288.42	11.8	Quantifier	289.2	97.1	78	35	3	1.0
			Qualifier	289.2	109.1	78	35	5	
^{13}C2-testosterone	290.41	11.8	ISTD	291.4	111.1	74	35	5	
17OHProgesterone	330.46	12.0	Quantifier	331.1	97.0	70	40	3	0.9
			Qualifier	331.1	109.3	70	45	5	
d8-17OHprogesterone	338.46	11.9	ISTD	339.5	100.1	50	45	7	
DHEA	288.42	12.1	Quantifier	271.3	197.2	55	25	3	3.0
			Qualifier	271.3	213.3	55	25	4	
Progesterone	314.46	12.8	Quantifier	315.6	97.1	80	30	3	1.1
			Qualifier	315.6	109.1	80	40	4	

Table 2.3: Analytes and ISTD compound-dependent parameters.

iii. Optimization of flow injection analysis parameters

At this stage of method development, physical source parameters are optimized to maximize the ionization efficiency of each analyte and ISTD. Since these parameters are used for all the analytes and ISTD, they were chosen according to a compromise in terms of different ionization efficiency of each compound. Small analytes amounts were repeatedly injected through the autosampler; the experiment was repeated with both the ionization sources, ESI and APCI. The parameters giving the maximum signal were chosen, evaluating the signal-noise ratio (S/N) of every peak. The best values were obtained with the APCI ionization with positive ion mode. All the parameters selected are in the table 2.4.

Nebulizer Current	3µA
Temperature	400°C
Curtain Gas	30 psi
Nebulizer Gas	30 psi
CAD Gas (nitrogen)	10mTorr

Table 2.4: Ion source parameters.

2a. Chromatography development

i. Chromatography system: HPLC Serie 200

The HPLC system was a Serie 200 PerkinElmer (PerkinElmer, Waltham, MA) equipped with two micro pumps, a quaternary pump, column oven, autosampler with samples tray, injector and a *peltier* temperature controller. The analytic column was a reverse phase Luna RP-C8, 100x4.6mm, 5µm, 100 Å suitable for low polarity analytes such as steroids; the guard column was RP-C8 4x2mm, 5µm both from Phenomenex (Torrance, CA, USA). As mobile phases methanol and water gradient grade were used, since they have the polarity suitable to achieve the separation of these analytes.

ii. LC analytes separation results

All the steroids included in the method were structurally very similar; some of them (11deoxycortisol/corticosterone; DOC/17OHprogesterone; testosterone/DHEA) were isobar sharing the same molecular weight. Frequently, isobar molecules can produce fragments with the same m/z: the only way to avoid the overlap of different fragments originated from a different parent ion is to separate them modulating the chromatography gradient, allowing to reach the revelator at different moments. Chromatographic resolution was achieved for all the isobaric steroid pairs and even for the steroids, like testosterone and androstenedione, which differ by only 2 atomic mass unit (amu) in their molecular weight.

iii. Analytic gradient development

The mobile phase A was 20% methanol and the mobile phase B was 100% methanol. The total flow rate was set at 750µl/min, suitable for APCI ionization and to reduce back-pressure with that kind of analytic column, and the column compartment temperature was 40°C. We optimized a gradient modulating the flow rate and composition; a good resolution for all the nine compounds included in the method was achieved in 21 minutes, paying particular attention to the separation of the isobar compounds. The elution was performed using increasing percentage of the organic phase. The gradient is represented in table 2.5 and the chromatogram in figure 2.13.

Time (min)	Flow (µl/min)	Solvent A (%)	Solvent B (%)
0	750	98.0	2.0
2.3	750	98.0	2.0
2.4	750	55.1	44.9
8.5	750	55.1	44.9
12.5	750	0.0	100.0
14.5	750	0.0	100.0
14.6	750	98.0	2.0
21.0	750	98.0	2.0

Table 2.5: Chromatography gradient.

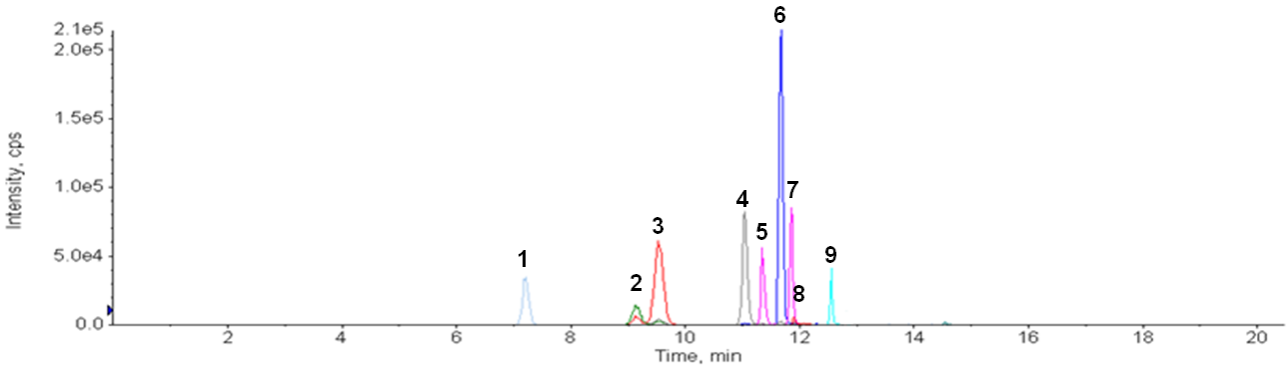


Figure 2.13: The chromatogram. (**1**: cortisol; **2**: corticosterone; **3**: 11deoxycortisol; **4**: androstenedione; **5**: DOC; **6**: testosterone; **7**: 17OHprogesterone; **8**: DHEA; **9**: progesterone)

iv. On-line purification assessment

To further improve the chromatographic process eliminating interfering substances, an on-line purification system was developed using a perfusion column POROS R1/20 2.1mmx30mm by Applied Biosystems (Foster City, CA). The POROS preparative column was placed before the analytic column thus obtaining a *two-dimensional chromatography*. To purify the sample at the preparative column, a flow from two lines of the quaternary pump was used: the solvent A of the quaternary first line was H_2O with 10% of methanol, while the flow of the second line B was 100% methanol. The sample was first loaded in the POROS column with a flow rate of 3ml/min of solvent A: the analytes were adsorpted by the internal POROS polymer while the high flow removed interfering samples molecules carrying them to the waste. After 1 minute, the micro pumps back-flush allowed the analytes elution from the POROS to the analytic column. The different flow coordination is organized through a ten-port switching valve (VICI, Houston, TX) connected with all the devices. The valve can assume two different positions: in the initial position A (figure 2.14-A) the flow of the quaternary pump goes into the valve from the port 3 loading the sample into the POROS (port 2) and going then to the waste through the port 8 and 9. After the first minute, the valve changes the position (position B, figure 2.14-B): the quaternary pump flow is directed to the waste while the flow of the micro pumps carries the analytes from the POROS (port 9 and 10) to the analytic column (port 1 and 2) allowing their chromatographic separation. After 16 minutes, the valve switch to the A position allowing the POROS cleaning by the quaternary solvent B and the re-equilibration of the analytic column by the micro pumps for the last 5 minutes.

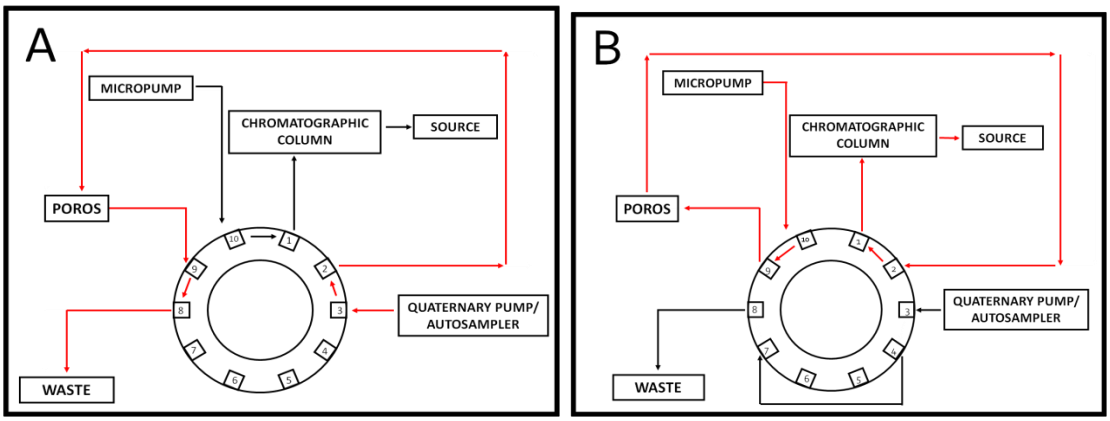


Figure 2.14: VALCO A and B position

3a. Pre-analytical phase: optimization of samples preparation

i. Blood withdrawals

The blood was collected in Vacuum Z serum beads clot activator. The samples were centrifuged soon after the withdrawal at 2000 x g for 10 minutes at room temperature and then sera were stored in 1.5 ml polypropylene tubes and frozen at -20°C until the analysis.

Subjects were saline-infused for 10 min before blood withdrawal to avoid stress-related alterations in the glucocorticoids cascade.

ii. Standard solutions, internal standard and calibrators

Stock solutions were prepared in methanol for each standard and ISTD at different concentrations in the mg/ml range. Working solutions were prepared at 100µg/ml for cortisol, progesterone and all the ISTD while for the other analytes were prepared 10µg/ml standard solutions. A stock calibrator was prepared by mixing all the analytes obtaining a solution with the following concentrations: cortisol, 500 ng/ml; DHEA and progesterone, 50 ng/ml; corticosterone, androstenedione and testosterone, 20 ng/ml; 11deoxycortisol, DOC and 17OHprogesterone, 10 ng/ml. The BSA 4% in H_2O was chosen as matrix for an eight-point calibration curve prepared by serial dilution from the stock calibration solution described above. The pure BSA solution represented the “zero point”. A working ISTD solution was prepared obtaining the following concentrations: d4-cortisol 50 ng/ml, d8-corticosterone and d2-11deoxycortisol 5 ng/ml, d2-DHEA 3 ng/ml,

13C2-testosterone 2 ng/ml and d8-17OHprogesterone 1 ng/ml, d9-progesterone 10 ng/ml. All the stock solution, working solution and calibrators were stored at -20°C.

iii. In-house and European quality controls

For the method validation three *in-house* quality controls (QCs) were created by generating serum pool mixing male serum, female serum and mixed sera adding pure standards to achieve three different concentration ranges for each analyte: low, medium and high. Aliquots of 1 ml of each pool were then frozen and three aliquots for every concentration range were thawed at room temperature and analyzed at the beginning, in the middle and at the end of every analysis batch to test the repeatability of the method *intra* and *inter* assay. To verify the accuracy of the analytic method, European certified sera (previously measured using GC/MS by the manufacturer company) were analyzed as unknown samples in each analysis batch.

iv. Samples purification optimization

After some tests, we developed a SPE method finding a compromise between sample washing, to avoid contaminants and analytes recovery percentage. For each curve calibrator, QCs and serum sample 900µl were transferred into a 12x75mm glass tube. To each tube was then added 1ml of deproteinization solution, prepared as follow:

- Solution A: water with 8,9% of $ZnSO_4$ $(H_2O)_7$

- Solution B: Solution A with methanol 1:5

For each ml of final solution 1µl of ISTD stock solution was added, containing the following concentrations of each ISTD: d4-cortisol, 50ng/ml; d8-corticoterone 5ng/ml; 11deoxycortisol, 5ng/ml; $^{13}C_2$-testosterone, 2ng/ml; d8-17OHprogesterone, 1ng/ml. The tubes were then vortexed for 3 minutes and centrifuged at 2000 x g for 10 minutes at room temperature: supernatants were transferred into the SPE cartridge previously activated for the extraction. The SPE procedure was performed as follow: 1ml of methanol to activate the C18 cartridge then conditioned with 1ml of H_2O; samples loading; washing with 3ml of H_2O and then elution with 1ml of methanol 100%. The eluted samples were collected in the 12x75mm glass tubes and then dried under a gentle stream of nitrogen. The samples were then reconstituted with 150µl of water and 150µl of methanol and transferred into autosampler vials and 200µl were injected in the LC-MS/MS system.

4a. LC-MS/MS quantification by isotopic dilution

The software used for the quantification of the mass spectrometry analysis and data processing was Analyst 1.5 by AB Sciex. The isotopic dilution quantification method by linear regression was the chosen quantification mode. This kind of quantification is based on the use of an *internal standard* (IS), a chemical compound added in known and constant amount to each calibrator, QC and sample. It must have the same physical and chemical characteristics of the analyte to measure; indeed, it is used to correct analytes loss during the sample preparation and sample injection, minimizing the analytes quantification variability. Since the IS must to be very similar but not identical to the analyte, the deuterated corresponding molecules are the most used. The minimum difference of molecular weight, depending on the number of substituted hydrogens, allows distinguishing the two compounds during the LC-MS/MS analysis.

The calibration curve was therefore built using the ratio between the pure standard and their correspondent IS on the y axis and the theoretic concentrations of the calibrators on the x axis. The concentration of each analyte was calculated by interpolation on the respective regression curve. Since the specific ISTD was not available for each analyte, the quantification was performed as follow: d4-cortisol for cortisol quantification; d8-corticosterone for corticosterone quantification; d2-11deoxycortisol for 11deoxycortisol quantification; $^{13}C_2$-testosterone for testosterone, androstenedione and DHEA quantification; d8-17OHprogesterone for 17OHprogesterone, DOC and progesterone quantification. The ISTD d5-testosterone, d2-DHEA and d9-progesterone were finally not used for the quantification because of their deuterium instability during the APCI ionization.

Vb. Assessment of quantification method validation

i. How to validate a quantitative analytical chemistry technique

There are some parameters that must be assessed for the validation of a quantitative analytical chemistry method defined by FDA[45]. Of course, they strictly depend on the compatibility between the analyte to measure and the technique/instrument of choice, and for this reason are completely different among laboratories.

- *Sensitivity*: the smallest concentration of a substance that can be reliably measured by the analytical method. For instrumental analysis, to define mathematically the sensitivity is used the *limit of detection* (LOD), the lowest analyte amount that can be distinguished from the absence of that substance (a *blank value*) and the *limit of quantification* (LLOQ), the lowest analyte amount that can be reasonably quantified with statistic certainty. Moreover, another parameter evaluated for the sensitivity of each peak can be the S/N, commonly used in mass spectrometry analysis. It is the ratio between the analyte signal and the background, representing how much the peak is distinguished from the basal noise.

- *Specificity*: the method capability to distinguish unequivocally the analyte among other substances present in the sample. The specificity is an important value especially when the quantification is in a complex biological matrix, such as plasma.

- *Accuracy and precision*: the accuracy is the proximity degree of a measured analytes amount to the true analytes amount in the matrix and is expressed as the ratio between the found value and the true value in percentage; the precision, also called *repeatability*, is the evaluation of how much repeated measurements under the same conditions show the same results. It can be calculated "*intra-assay"* when the repeated measurements are made during the same day and the same analytical session and "*inter-assay"* when they are made in different days.

All these parameters together, carefully evaluated during the method validation, can provide significant information to drive the method development and eventual targeted technical improvements.

ii. Determination of LC-MS/MS sensitivity and linearity

Linearity was achieved for three or four orders of magnitude, depending on the analyte. The LOD was calculated as the lowest concentration exhibiting a S/N above 3, while the LLOQ was calculated as the lowest quantifiable concentration with a S/N higher than 10, an accuracy between 80 and 120% of the true value and a CV (coefficient of variation) lower than 20%. The LOD and LLOQ evaluation was made on five replicates of the same injection. The sensitivity was also investigated adding known low amount of the analytes to the steroid-free serum. All the data obtained are summarized in the table 2.6.

	Linearity	**r^2**	**LLOQ (pure std)**				**LOD (pure std)**	**Serum Sensitivity**
	ng/ml		**ng/ml**	**S/N**	**CV%**	**Accuracy %**	**pg**	**ng/ml**
Cortisol	0.244 – 500.0	0.9997	0.2440	49	9.0	99.3	4.8	0.244
Corticosterone	0.039 – 20.0	0.9995	0.0391	13	3.4	95.9	4.0	0.313
11Deoxycortisol	0.019 – 10.0	0.9993	0.0195	14	15.3	94.1	2.0	0.078
Androstenedione	0.019 – 20.0	0.9998	0.0195	14	13.2	100.7	2.3	0.039
DOC	0.019 – 10.0	0.9994	0.0195	11	9.6	107.8	2.5	0.078
Testosterone	0.019 – 20.0	0.9993	0.0195	11	6.2	94.2	2.8	0.019
17OHProgesterone	0.010 – 10.0	0.9996	0.0098	11	8.9	107.5	1.7	0.078
DHEA	0.195 – 50.0	0.9995	0.1953	10	11.2	97.4	29.2	0.781
Progesterone	0.024 – 50.0	0.9999	0.0244	29	9.3	103.4	1.2	0.049

Table 2.6: Method sensitivity and linearity.

iii. Specificity assessment

As mentioned before, endogenous and exogenous steroid hormones are compounds structurally very similar. The potential interference of the following compounds was therefore evaluated: 21deoxycortisol, epitestosterone, DHEA-S, cortisone, prednisone and prednisolone, triamcinolone acetonide, methylprednisolone, dexamethasone and betamethasone. The absence of these compounds interference was confirmed: some of these molecules had the same m/z and fragmentation pattern of analytes included in the method but they were separated with chromatography. The specificity was further evaluated with

the analyte *ion ratio* (IR): the ratio between the quantifier and qualifier ion peak area was calculated for each analyte in each sample and if the ratio differed more than 20% from the respective pure standard IR, the measurement was considered not validable.

iv. Determination of accuracy and precision

The accuracy was calculated as described before and accepted between the 80 and 120%. It was evaluated through European certified sera measurements for the analytes cortisol, testosterone, 17OHprogesterone and progesterone. For the others European certified sera were not commercially available, therefore the accuracy was evaluated by *in-house* validation, spiking gravimetrically determined quantities of pure standards in steroid-free serum. We calculated a *low*, *medium* and *high* range different for each analytes, based on physiological ranges. The accuracy obtained for each analyte at low, medium and high concentration range is reported in table 2.7.

The intra-assay precision (accepted <15%) was estimated by measuring six replicates of serum samples with low, medium and high concentration range. The inter-assay precision (accepted <20%) was calculated evaluating six replicates of the three concentration ranges of six different days in six different weeks. The results are in table 2.7.

	Low Range			Medium Range			High Range		
	Accuracy %	Intra-assay CV%	Inter-assay CV%	Accuracy %	Intra-assay CV%	Inter-assay CV%	Accuracy %	Intra-assay CV%	Inter-assay CV%
Cortisol	94.9	3	8	103.7	3	7	93.6	2	5
Corticost.	92.5	2	10	98.3	6	5	98.5	3	7
11Deoxycor.	106.2	8	8	99.1	4	5	102.3	3	2
Androst.	86.3	10	11	97.9	10	10	100.6	7	11
DOC	100.7	5	6	100.0	6	9	104.1	6	9
Testost.	99.9	4	7	97.4	4	7	99.6	3	4
17OHProg.	104.4	5	9	102.1	4	5	101.4	4	5
DHEA	94.6	8	8	101.8	7	9	98.2	8	10
Prog.	83.7	8	11	92.4	5	6	92.0	5	6

Table 2.7: Method accuracy at three different concentration ranges

v. Evaluation of matrix effect

Even after several purification steps, the presence of other molecules in the sample can alter the ionization efficiency of the analytes either enhancing or decreasing the signal. In mass spectrometry analyses, this effect is called *matrix effect*. The typical matrix effect consists in a decrease of the signal through a mechanism called *ion suppression*, due to the competition between the analyte and other molecules for the ionization. Good sample purification and the use of ISTD minimize consequences of the eventual matrix effect, avoiding a sensitivity decrease.

The matrix effect during the development of this method was evaluated at two levels. First, known amounts of pure analytes were added to the steroid-free serum and BSA after the extraction; the samples were then injected in the LC-MS/MS system and the ratio between the signal and the signal obtained by injecting pure analyte was calculated, to verify whether the presence of the matrix was altering the analyte signal. Second, a mix of the nine pure analytes was injected directly in the ionization source while a blank matrix sample was injected from the HPLC system. Thus, the stable signal given by the pure analytes injected in the source can be enhanced or decreased by the matrix. Alterations of the analytes signal were not observed. All the results concerning the matrix effect experiments are in table 2.8.

	Matrix Effect in BSA (recovery %)	**Matrix Effect in serum (recovery %)**
Cortisol	84.8	87.4
Corticosterone	96.8	95.5
11Deoxycortisol	92.8	96.1
Androstenedione	98.3	99.0
DOC	97.4	96.5
Testosterone	97.3	99.1
17OHProgesterone	98.3	100.9
DHEA	101.2	103.8
Progesterone	100.2	100.6

Table 2.8: Evaluation of matrix effect.

Vc. Results of quantification methods comparison study: immunoassay vs LC-MS/MS

The comparison study has been carried out for six of the nine steroid hormones included in our quantification method, since corticosterone, 11deoxycortisol and DOC were not measured by the Laboratorio Centralizzato.

First, the sensitivity of the two quantification methods was compared (table 2.9). For cortisol, testosterone, progesterone, androstenedione and 17OHprogesterone the LC-MS/MS method achieved the best sensitivity, whereas for DHEA the best sensitivity value was achieved by immunometric assays. By Mann-Whitney comparison, there were no statistically significant differences for testosterone in males and progesterone above 1 ng/ml. Lower median values were obtained by LC-MS/MS for cortisol (-16%, $p = 0.0052$), testosterone in females (-26%, $p = 0.0080$) and progesterone below 1 ng/ml (-84%, $p < 0.0001$). Lower measurements ($p < 0.0001$) were provided by LC-MS/MS also for the analytes androstenedione, DHEA and 17OHprogesterone. A good correlation, with a *correlation coefficient* (r) above 0,9, was obtained for cortisol, male testosterone, progesterone above 1 ng/ml, androstenedione and DHEA; lower correlation grade was obtained for females testosterone (r=0.773), progesterone below 1 ng/ml (r=0.637) and 17OHprogesterone (r=0.874). In figure 2.15 are represented the Deming regression graphs, in which results obtained with LC-MS/MS are plotted on the x-axis and results obtained with immunometric assays are on the y-axis. The dotted line represents the perfect correspondence between the two quantitative methods, with a theoretical slope and intercepts values respectively of 1 and 0, while the black line represents the real obtained correspondence. The perfect methods correspondence (slope=1; intercept=0) was obtained for testosterone in males. Slope values higher than 1 were achieved for cortisol and progesterone >1ng/ml (confidence interval, CI 95%: 1.119 – 1.233; 1.077 – 1.378 respectively). Testosterone in females and progesterone below 1 ng/ml showed elevated slope coefficients (95%CI: 1.311–2.137 and 0.253–6.149, respectively) and testosterone in females had also a negative intercept coefficient (95%CI: −0.326 to −0.036). Significantly elevated slopes were obtained also for androstenedione, DHEA and 17OHprogesterone (95%CI: 2.068–2.530; 2.348–3.068; 0.870–1.589, respectively).

A Bland Altman analysis of the data was performed: on the y-axis is shown the percentage difference between results obtained by immunoassay and LC–MS/MS on average; on the x-axis the average results of the two methods. All the obtained data are in figure 2.16. An acceptable mean difference was achieved for males and females testosterone, cortisol and progesterone >1ng/ml. Among them, only for males testosterone the difference was not different from 0% (95%CI: -6.6% to 1.5%). Mean differences significantly different from 0, ranging between 80.2% and 133.2%, were obtained for androstenedione, 17OHprogesterone, DHEA and progesterone <1ng/ml. Furthermore, for these analytes the agreement intervals were located in the positive part of the graphs. These data indicated a good accuracy and calibration of the immunometric platform ElecsysE170, used for the determination of cortisol, testosterone

and progesterone. However, the data showed a possible cross-reactivity at low concentrations for testosterone and progesterone.

Concluding the comparative analysis, calibrators of 17OHP Bridge, Immulite2000 and DSL9000 were measured with the LC-MS/MS method as unknown samples. Results showed a good accuracy for 17OH Bridge calibrators (92.8 – 101.6%) confirming that the low correlation between the two methods for the 17OHprogesterone measurement is due to a low specificity of the immunoassay. A calibration problem was found for the DSL9000 used for DHEA measurement: the calibrator's accuracy was between 46.2 and 55.5%, explaining the overestimation of this analyte. For the androstenedione measurement, Immulite2000 calibrators showed an accuracy of 133.1% for low levels (0.5ng/ml), and 82.5% for high levels (5ng/ml), explaining, also in this case, the analyte overestimation.

SENSITIVITY	LC-MS/MS in serum (ng/ml)	Immunoassays (ng/ml)
Cortisolo	0.244	1.000
Androstenedione	0.039	0.300
Testosterone	0.019	0.100
17OHProgesterone	0.078	0.100
DHEA	0.781	0.200
Progesterone	0.049	0.100

Table 2.9: RIA and LC-MS/MS sensitivity comparison.

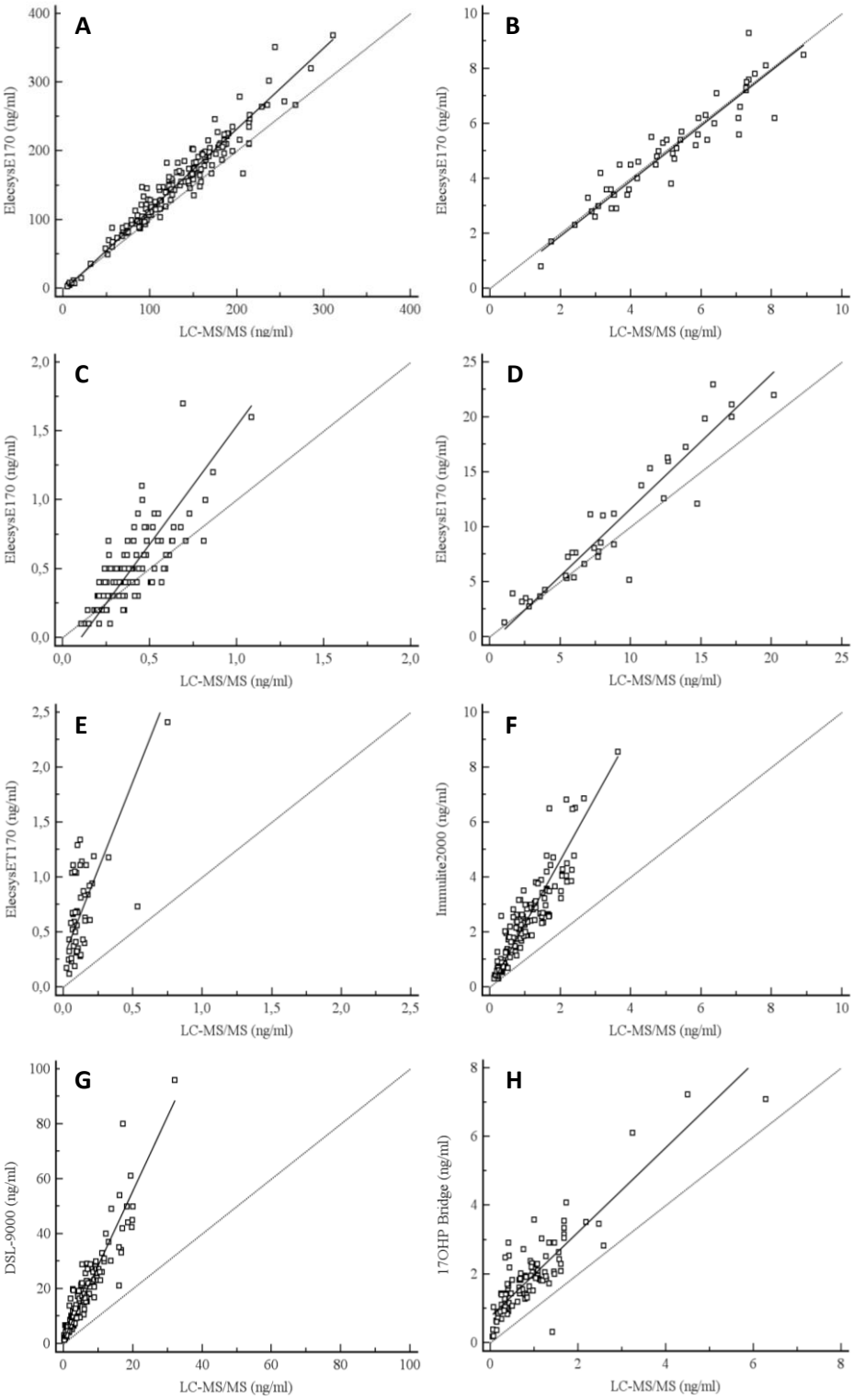


Figure 2.15: Deming regression analysis: cortisol (A: m=1.176±0.029; q=−3.093±3.147; r=0.968), males testosterone (B: m=1.004±0.062; q=−0.097±0.283; r=0.938), females testosterone (C: m=1.724±0.208; q=−0.181±0.073; r=0.773), progesterone >1ng/ml (D: m=1.227±0.074; q=−0.587±0.573; r=0.946), progesterone <1ng/ml (E: m=3.201±1.466; q=0.270±0.166; r=0.637), androstenedione (F: m=2.299±0.117; q=0.042±0.093; r=0.906), DHEA (G: m=2.708±0.182; q=1.478±0.800; r=0.929) and 17OHprogesterone (H: m=1.230±0.181; q=0.769±0.152; r=0.874); m: slope; q: intercept.

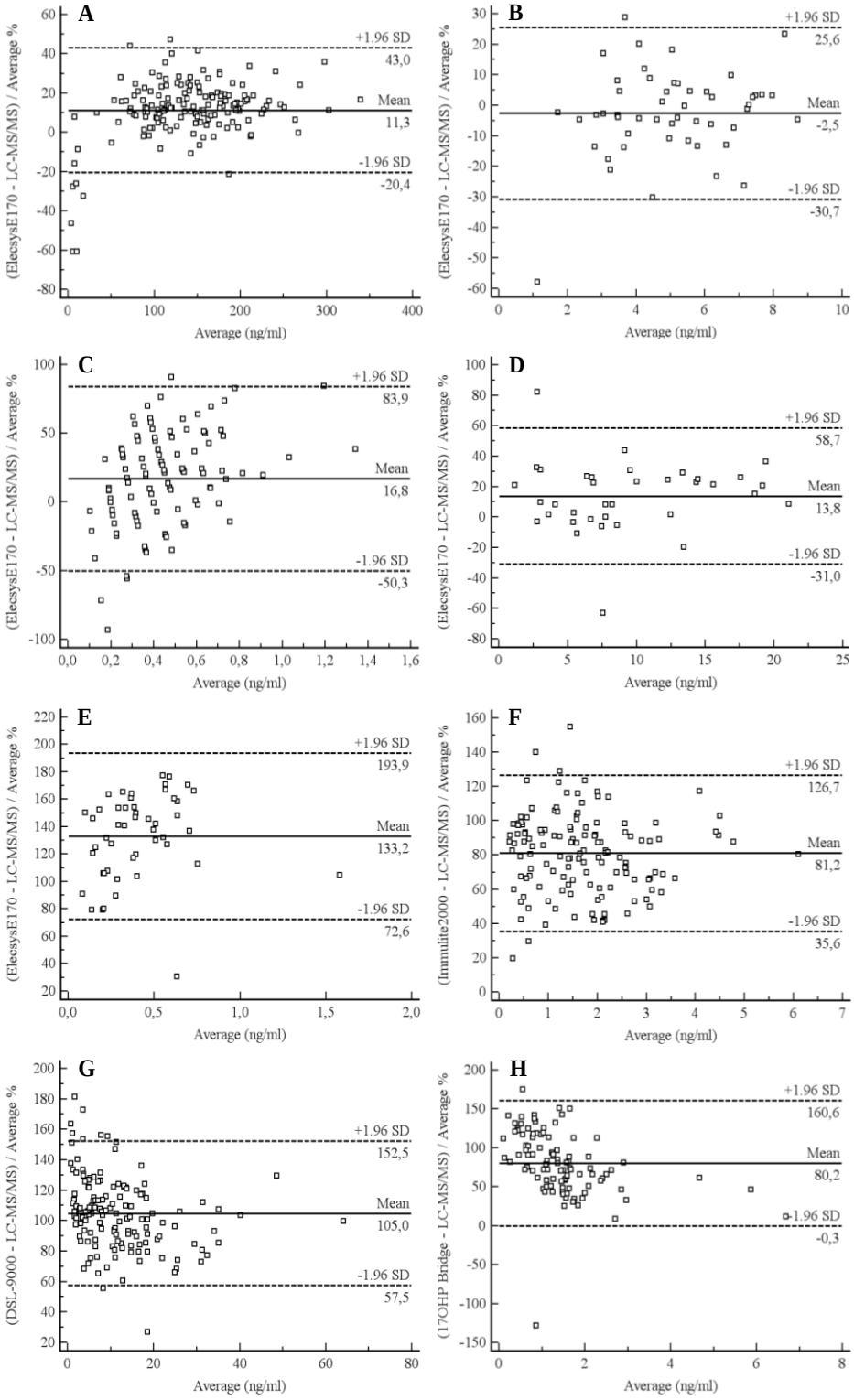


Figure 2.16: Bland Altman analysis: A – cortisol; B – males' testosterone; C – females testosterone; D – progesterone >1ng/ml; E – progesterone <1ng/ml; F – androstenedione. Y axis: percentage of the difference between RIA result and LC-MS/MS result compared to the average; x axis: average of the two methods results.

Vd. Estimation of steroids reference intervals

356 healthy adults were grouped according to sex and age, obtaining five groups for males (18-29, 30-39, 40-49, 50-59, ≥60 years old) and five groups for women (18-29, 30-39, 40-49, 50-59, ≥60 years old). Anthropometric subjects characteristics are reported in table 2.10; reference intervals are reported in table 2.11.

Kruskal Wallis analysis showed results significantly different among the age groups for BMI and waist circumference, with higher values in the groups 50-59 and >60 for both genders (females: $p=0.036$ and $p=0.018$; males $p=0.0009$ and $p<0.0001$ respectively). Similarly, higher values of cholesterol ($p<0.0001$ for both genders), PAD ($p=0.0065$ for females and $p=0.0063$ for males) and PAS ($p<0.0001$ for females and $p=0.0004$ for males) were found in the older people group for both genders while higher value of HDL was found for males ($p=0.0006$) and higher value of triglycerides was found in women ($p<0.0001$). Furthermore, different characteristics of females in luteal and follicular phase are reported in table 2.12: significant differences were not observed for anthropometric characteristics in these two groups.

The LC-MS/MS method was sensitive enough to determine circulating levels in the healthy adult population of eight analytes: cortisol, corticosterone, 11deoxycortisol, androstenedione, testosterone, DHEA, 17OHprogesterone and progesterone. Most of the samples showed a concentration lower than the sensitivity in serum matrix for the DOC (0.078 ng/ml) therefore the reference range was not calculated for this analyte. One value of corticosterone, testosterone and 17OHprogesterone, two of DHEA, four of 11deoxycortisol and 28 of progesterone were discarded because the IR values were not accurate. Values below the sensitivity limit were found for some analytes: one for corticosterone, androstenedione and DHEA, 13 for 11deoxycortisol, 3 for 17OHprogesterone and 65 for progesterone (71% of them in the post-menopausal subgroup) in the female group and 4 measurements of 11deoxycortisol and 39 of progesterone in males group.

Analyzing the steroid profile with Kruskal Wallis analysis, we found significant differences for DHEA and progesterone among the different age groups for both males ($p<0.0001$) and women ($p<0.0001$), showing an age-dependent tendency to decrease. The same tendency was observed for androstenedione ($p<0.0001$), testosterone ($p<0.0001$) and 17OHprogesterone ($p<0.0001$) in women. Males testosterone showed a not significant ($p=0.086$) tendency to decrease with age. Among women in different menstrual cycle phases, 17OHprogesterone and progesterone were significantly higher in luteal phase ($p<0.0001$). A non significant tendency to increase in the luteal phase was found for cortisol and corticosterone.

A	F 18-29 n=31		F 30-39 n=41		F 40-49 n=56		F 50-59 n=36		F ≥60 n=13	
	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P
Age (years)	27.0	18.0 - 29.0	35.0	30.5 - 39.0	45.0	40.0 - 50.0	55.0	51.0 - 59.0	64.0	60.0 - 90.0
BMI (kg/m^2)	21.2	18.8 - 24.8	21.2	18.8 - 24.6	21.7	18.1 - 24.8	22.8	19.4 - 25.0	23.1	18.1 - 24.7
Waist Circum. (cm)	76.0	65.6 - 83.72	76.0	64.5 - 86.9	76.0	63.7 - 87.0	79.5	66.4 - 87.2	83.0	67.0 - 87.0
SBP (mmHg)	70.0	60.0 - 80.0	77.5	60.2 - 80.0	80.0	60.0 - 90.0	80.0	70.0 - 90.0	80.0	70.0 - 85.0
DBP (mmHg)	110.0	95.0 - 120.0	110.0	100.0 - 129.5	115.0	95.3 - 140.0	120.0	105.0 - 140.0	135.0	110.0 - 160.0
Total cholesterol (mg/dl)	149.5	108.0 - 202.2	170.0	129.5 - 254.2	188.5	148.9 - 279.1	193.5	138.1 - 285.8	204.0	155.0 - 260.0
HDL (mg/dl)	59.5	22.0 - 87.8	50.0	29.6 - 87.2	57.5	33.7 - 103.2	59.5	32.4 - 88.6	63.0	45.0 - 89.0
Triglycerides (mg/dl)	49.5	27.3 - 90.6	56.0	35.9 - 108.6	60.0	38.5 - 113.2	66.0	28.9 - 130.8	110.0	57.0 - 137.0
Glycemia (mg/dl)	75.5	44.2 - 98.4	78.5	59.4 - 107.4	82.0	49.9 - 103.3	82.0	61.0 - 100.3	79.0	61.0 - 105.0
Insulin (µU/ml)	6.10	3.35 - 13.45	5.05	2.29 - 12.44	4.10	1.89 - 13.21	5.70	3.24 - 9.55	4.40	2.80 - 14.00

B	M 18-29 n=49		M 30-39 n=44		M 40-49 n=38		M 50-59 n=23		M ≥60 n=26	
	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P
Age (years)	24.0	18.0 - 29.0	36.0	30.0 - 39.0	44.5	40.0 - 49.0	52.0	50.0 - 59.0	66.0	60.0 - 87.7
BMI (kg/m^2)	22.3	18.8 - 24.9	23.2	19.2 - 25.0	23.6	19.2 - 25.0	23.9	20.7 - 25.0	24.0	21.2 - 25.0
Waist Circum. (cm)	79.0	68.2 - 92.6	84.0	69.4 - 92.4	85.5	74.7 - 96.1	91.0	78.0 - 97.0	88.0	77.1 - 98.9
SBP (mmHg)	80.0	70.0 - 90.0	75.0	65.0 - 90.0	80.0	65.0 - 100.0	85.0	75.0 - 90.0	80.0	60.1 - 89.9
DBP (mmHg)	120.0	100.0 - 130.0	120.0	100.0 - 140.0	110.0	105.0 - 130.0	125.0	110.0 - 150.0	130.0	120.0 - 150.0
Total cholesterol (mg/dl)	148.5	108.9 - 261.5	182.0	139.4 - 261.1	181.0	142.6 - 257.7	194.0	157.7 - 262.9	198.5	99.8 - 235.0
HDL (mg/dl)	38.0	25.7 - 93.5*	43.0	28.7 - 71.6	41.5	26.6 - 63.0	48.5	30.1 - 81.5	56.0	25.1 - 95.8
Triglycerides (mg/dl)	67.0	33.4 - 179.8	62.0	36.9 - 168.1	76.3	48.5 - 155.9	79.0	52.2 - 141.6	71.0	46.5 - 165.1
Glycemia (mg/dl)	77.0	56.4 - 102.0	81.5	64.0 - 102.0	81.0	61.2 - 99.0	88.0	63.2 - 101.9	85.5	59.3 - 98.9
Insulin (µU/ml)	6.40	2.50 - 14.62	5.00	2.25 - 11.66	5.57	2.05 - 12.67	6.10	2.56 - 9.78	4.65	2.31 - 10.83

Table 2.10: Anthropometric and metabolic characteristics of the healthy subjects: females (**A**) and males(**B**).

A	F 18-29 n=31		F 30-39 n=41		F 40-49 n=56		F 50-59 n=36		F ≥60 n=13	
	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P
Cortisol	109.7	62.3-256.4	108.6	57.8-189.3	100.5	52.0-198.1	121.5	57.4-186.3	120	56.9-180.8
Corticosterone	3.59	0.52-14.38	3.320	0.93-11.01	2.88	0.67-17.71	3.64	0.66-9.91	3.47	0.62-8.96
11Deoxycorisol	0.301	<1.015	0.211	<1.085	0.251	<0.998	0.3	<0.786	0.311	0.118-0.782
Androstenedione	0.849	0.376-2.070	0.760	0.434-1.722	0.572	0.200-1.068	0.349	0.137-0.791	0.222	0.039-0.632
DOC	0.078	0.078-0.142	0.078	0.078-0.091	0.078	0.078-0.178	0.078	0.078-0.114	0.078	0.078-0.101
Testosterone	0.267	0.111-0.411	0.251	0.116-0.490	0.213	0.105-0.409	0.160	0.081-0.414	0.166	0.045-0.634
17OHProgesterone	0.816	0.156-2.958	0.642	0.240-2.193	0.501	0.110-2.273	0.242	0.079-1.534	0.209	0.121-1.356
DHEA	6.35	2.25-26.74	4.670	1.58-17.49	3.470	1.09-15.03	2.685	1.06-7.27	1.800	<8.11
Progesterone	5.278	<20.302	0.699	<19.165	0.182	<15.360	<0.049	<7.419	<0.049	0.049-0.081

B	M 18-29 n=49		M 30-39 n=44		M 40-49 n=38		M 50-59 n=23		M ≥60 n=26	
	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P	Median	2.5 - 97.5 P
Cortisol	137.5	65.8-194.8	137.3	54.9-208.3	119.4	53.3-196.7	136.2	53.8-189.5	118.6	87.3-171.3
Corticosterone	4.33	0.92-12.29	4.17	0.64-17.68	2.94	0.72-16.68	3.89	1.58-9.62	3.99	0.96-9.15
11Deoxycorisol	0.432	0.079-1.188	0.373	<1.053	0.298	0.087-0.938	0.357	0.082-0.995	0.309	0.095-1.038
Androstenedione	0.627	0.353-1.345	0.595	0.229-1.321	0.596	0.311-1.108	0.549	0.395-1.188	0.449	0.278-0.753
DOC	<0.078	<0.147	<0.078	<0.138	<0.078	<0.159	<0.078	<0.078	<0.078	<0.144
Testosterone	5.439	1.452-10.174	5.623	3.075-8.299	4.878	2.134-7.177	5.231	2.264-7.896	4.292	2.635-7.205
17OHProgesterone	1.246	0.295-3.310	1.154	0.539-2.807	1.173	0.357-2.346	1.058	0.471-1.965	0.975	0.441-1.834
DHEA	7.76	2.93-16.33	5.76	2.03- 3.98	4.040	1.53-11.57	3.51	1.68-9.70	2.21	<6.66
Progesterone	0.093	<0.207	0.094	<0.225	0.084	<0.164	0.058	<0.116	0.051	<0.132

Table 2.11: Median and reference intervals of steroids levels in healthy subjects (ng/ml): females (**A**) and males(**B**).

	Follicular Phase, n=45		Luteal Phase, n=21	
Characteristics	**Median**	**2.5 - 97.5 P**	**Median**	**2.5 - 97.5 P**
Age (years)	41.0	21.9 - 49.4	37.0	18.0 - 52
BMI (kg/m^2)	21.8	18.9 - 24.7	21.8	17.9 - 24.1
Waist Circum. (cm)	76.0	66.9 - 86.4	76.0	65.0 - 86.0
SBP (mmHg)	80.0	60.0 - 90.0	80.0	60.0 - 80.0
DBP (mmHg)	110.0	100.0 - 133.6	110.0	90.0 - 140.0
Total cholesterol (mg/dl)	179.0	130.2 - 235.4	176.0	133.2 - 246.0
HDL (mg/dl)	58.0	31.7 - 95.4	56.0	35.3 - 88.9
Triglycerides (mg/dl)	56.0	36.6 - 91.2	60.0	40.0 - 100.8
Glycemia (mg/dl)	79.0	46.9 - 100.3	80.0	53.0 - 92.0
Insulin (μU/ml)	4.10	2.20 – 9.84	6.20	3.10 - 11.90
Steroids	**Median**	**2.5 - 97.5 P**	**Median**	**2.5 - 97.5 P**
Cortisol (ng/ml)	98.7	51.1 - 195.1	114.5	62.9 - 281.4
Corticosterone (ng/ml)	2.77	0.66 - 14.25	4.61$^{\#}$	1.16 - 15.80
11Deoxycortisol (ng/ml)	0.224	0.084 - 1.025	0.342	0.079 - 0.744
Androstenedione (ng/ml)	0.594	0.267 - 1.970	0.739	0.337 - 2.111
DOC (ng/ml)	<0.078	<0.088	0.078	<0.142
Testosterone (ng/ml)	0.210	0.108 - 0.421	0.245	0.102 - 0.401
17OHProgesterone (ng/ml)	0.382	0.139 - 1.100	1.587$^{\#\#}$	0.235 - 2.959
DHEA (ng/ml)	4.20	1.15 - 17.63	4.48	1.68 - 18.29
Progesterone (ng/ml)	0.103	<1.707	10.394$^{\#\#}$	0.052 - 25.830

Table 2.12: Anthropometric and metabolic characteristics of females in luteal and follicular phase and relative steroids reference intervals. ($^{\#}$ p<0.05; $^{\#\#}$ p<0.0001).

Ve. Evaluation of steroid profile in obese subjects

Steroids levels were then determined in three subgroups of subjects : normalweight subjects (NW), overweight subjects (OW) and obese subjects (OB). The anthropometric characteristics of these subjects are summarized in table 2.13. In both genders, overweight and obese subjects showed higher waist circumference, PAD and PAS, insulin, glycemia and triglycerides and lower HDL compared to the normalweight subjects.

Concerning the circulating steroids profile (table 2.14), in females cortisol levels were significantly lower ($p=0.0032$) in the obese population; corticosterone levels decreased progressively from the normalweight to the obese group ($p=0.0081$). Significant differences were not found for other steroids. In males, on the contrary, significant differences were not found for glucocorticoids. The differences were found for 17OHprogesterone and testosterone ($p=0.0022$ and $p<0.0001$ respectively), both decreased in the obese group compared to overweight and normal weight population. Dunn's multiple comparison tests were performed to evaluate the specific significant differences among the three groups of subjects (figure 2.17-18).

A	NW n=59		OW n=54		OB n=58		Kruskal-Wallis
Characteristics	Median	IQR 25 - 75 P	Median	IQR 25 - 75 P	Median	IQR 25 - 75 P	p
Age (years)	48.0	41.0 - 58.0	48.0	42.0 - 60.0	48.0	40.0 - 58.0	n.s.
BMI (kg/m^2)	22.1	21.1 - 23.3	27.1	25.9 - 27.9	33.3	30.9 - 37.9	<0.0001
Waist Circum. (cm)	79.0	74.0 - 83.0	91.0	84.0 - 95.0	104.5	99.5 - 113.0	<0.0001
SBP (mmHg)	80.0	75.0 - 85.0	80.0	80.0 - 90.0	80.0	80.0 - 90.0	0.0109
DBP (mmHg)	120.0	110.0 - 130.0	130.0	120.0 - 142.5	130.0	120.0 - 142.5	0.0001
Total cholesterol (mg/dl)	5.30	3.80 - 6.90	6.85	6.00 - 8.80	11.65	8.40 - 14.60	<0.0001
HDL (mg/dl)	80.0	72.2 - 90.8	84.0	78.0 - 92.5	92.0	82.2 - 99.0	0.0003
Triglycerides (mg/dl)	193.0	169.7 - 225.2	200.0	178. 0 - 224.0	191.0	176.0 - 216.0	n.s.
Glycemia (mg/dl)	63.0	57.0 - 70.5	58.0	49.0 - 67.0	53.0	44.0 - 60.0	0.0003
Insulin (µU/ml)	65.0	51.2 - 82.5	78.5	58.0 - 111.0	82.5	65.0 - 121.0	0.0044

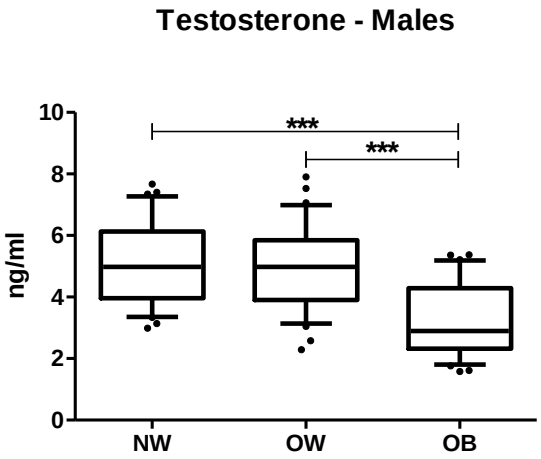
B	NW n=59		OW n=54		OB n=58		Kruskal-Wallis
Characteristics	Median	IQR 25 - 75 P	Median	IQR 25 - 75 P	Median	IQR 25 - 75 P	p
Age (years)	51.0	44.0 - 61.0	50.5	44.0 - 61.0	50.5	44.0 - 61.0	n.s.
BMI (kg/m^2)	23.3	22.1 - 24.2	27.1	25.7 - 28.7	32.8	31.2 - 36.2	<0.0001
Waist Circum. (cm)	87.0	83.0 - 90.0	98.5	95.0 - 102.0	112.0	108.0 - 119.0	<0.0001
SBP (mmHg)	80.0	80.0 - 85.0	85.0	80.0 - 90.0	90.0	80.0 - 100.0	0.0002
DBP (mmHg)	120.0	120.0 - 130.0	130.0	120.0 - 140.0	145.0	133.7 - 152.5	<0.0001
Total cholesterol (mg/dl)	5.10	3.80 - 6.60	8.10	6.00 - 11.00	15.0	10.40 - 21.30	<0.0001
HDL (mg/dl)	88.0	81.2 - 96.0	90.0	75.5 - 98.0	92.5	80.0 - 97.5	n.s.
Triglycerides (mg/dl)	201.5	180.0 - 221.0	208.5	191.0 - 228.0	202.5	183.0 - 240.0	n.s.
Glycemia (mg/dl)	56.5	43.0 - 63.0	48.5	40.0 - 54.0	40.5	36.0 - 47.0	0.0002
Insulin (µU/ml)	73.0	55.0 - 114.0	105.0	76.0 - 156.0	139.5	109.0 - 190.0	0.0001

Table 2.13: Anthropometric and metabolic characteristics of normo-weight (NW), over-weight (OW) and obese (OB) populations. Females (**A**) and males(**B**). (n.s. : not significant)

A	Median	IQR 25 - 75 P	Median	IQR 25 - 75 P	Median	IQR 25 - 75 P	p
Cortisolo (ng/ml)	112.0	97.4 - 146.2	111.1	83.5 - 143.5	92.0	72.8 - 108.9	0.0032
Corticost. (ng/ml)	3.43	1.66 - 6.78	2.79	1.74 - 5.86	2.03	1.27 - 3.86	0.0081
11Desoxycor. (ng/ml)	0.317	0.187 - 0.500	0.338	0.204 - 0.461	0.251	0.184 - 0.400	n.s.
Androst. (ng/ml)	0.550	0.316 - 0.705	0.483	0.405 - 0.760	0.485	0.324 - 0.672	n.s.
DOC (ng/ml)	<0.078	<0.078	<0.078	<0.078	<0.078	<0.078	n.s.
Testost. (ng/ml)	0.198	0.135 - 0.253	0.194	0.147 - 0.256	0.190	0.137 - 0.238	n.s.
17OHProg. (ng/ml)	0.378	0.211 - 0.500	0.423	0.253 - 0.665	0.322	0.203 - 0.666	n.s.
DHEA (ng/ml)	3.87	1.82 - 6.24	4.06	2.26 - 5.37	3.10	2.03 - 6.13	n.s.
Prog. (ng/ml)	0.060	<0.910	0.091	<1.059	0.049	<1.357	n.s.

B	Median	IQR 25 - 75 P	Median	IQR 25 - 75 P	Median	IQR 25 - 75 P	p
Cortisolo (ng/ml)	125.4	105.7 - 149.6	128.3	105.9 - 150.0	112.4	102.1 - 143.9	n.s.
Corticost. (ng/ml)	3.90	2.87 - 5.58	4.62	2.03 - 6.29	3.15	2.28 - 5.39	n.s.
11Desoxycor. (ng/ml)	0.385	0.232 - 0.545	0.415	0.278 - 0.551	0.409	0.272 - 0.562	n.s.
Androst. (ng/ml)	0.530	0.457 - 0.678	0.637	0.442 - 0.771	0.561	0.371 - 0.761	n.s.
DOC (ng/ml)	<0.078	<0.078	<0.078	<0.078	<0.078	<0.078	n.s.
Testost. (ng/ml)	4.981	3.974 - 6.055	4.979	3.937 - 5.789	2.898	2.363 - 4.281	<0.0001
17OHProg. (ng/ml)	1.078	0.884 - 1.527	1.031	0.868 - 1.269	0.811	0.640 - 1.063	0.0022
DHEA (ng/ml)	3.64	2.77 - 5.74	4.17	2.32 - 7.54	4.04	2.50 - 5.73	n.s.
Prog. (ng/ml)	0.061	<0.103	0.067	<0.095	0.056	<0.071	n.s.

Table 2.14: Circulating steroid hormones of normal weight (NW), overweight (OW) and obese (OB) populations respectively. Females (**A**) and males(**B**). (n.s. : not significant)



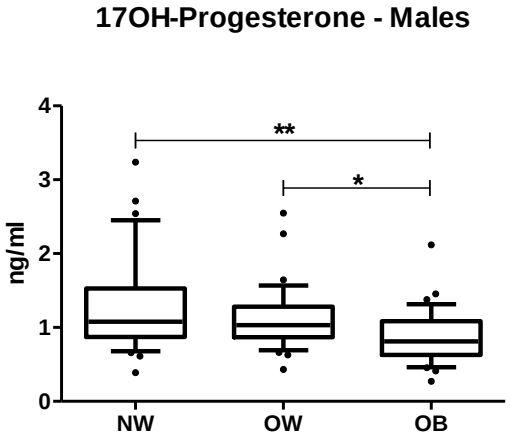
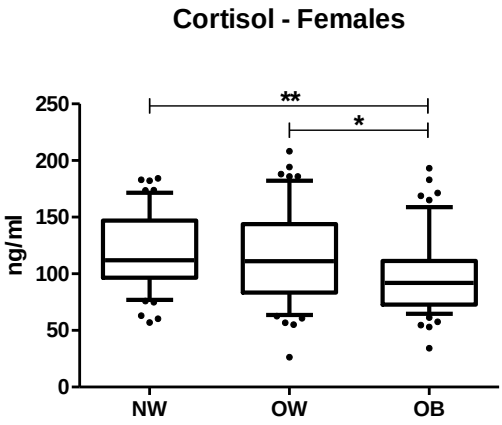


Figure 2.17: Dunn's Multiple Comparison Test performed for testosterone and 17OH progesterone in males.



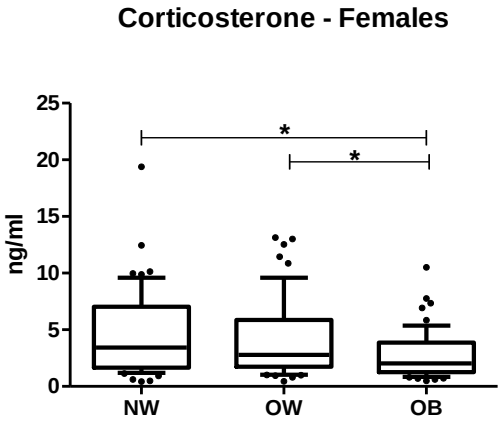


Figure 2.18: Dunn's Multiple Comparison Test performed for cortisol and corticosterone in females.

VI. Discussion and conclusion

The introduction of LC-MS/MS is the most important advance in endocrinology laboratories since the development of the immunoassays[95]. Since the well known limits of immunoassays and, on the other hand, the fast technical improvements of the liquid chromatography and mass spectrometry in the last decade, LC-MS/MS has improved the worth and the credibility of hormones quantification[13], offering sensitive, specific and multi-analytes measurements, reducing analysis time and cost per sample. For this reason, nowadays an increasing number of laboratories have started using this technology for routine clinical diagnostic measurements[98].

In this context, we developed a sensitive, specific and accurate multi-analytes quantification method for the quantification of nine serum steroids: cortisol, corticosterone, 11deoxycortisol, androstenedione, DOC, testosterone, DHEA, 17OHprogesterone and progesterone. These hormones have been chosen for their clinical relevance and, since our aim was to develop a multi-analytes method, chemical compatibility: other important steroids such as aldosterone, DHT, pregnenolone and the estrogens were not included in this method because they require a different kind of ionization and a different polarity. With a single chromatographic run of 21 minutes, we obtained the panel of nine circulating steroids, offering to the clinician a powerful tool for the diagnosis of steroids-related pathologies. By using a higher serum volume (900 µl) than those reported in other published multi-analyte methods[99-101], we were able to provide a general better sensitivity determined during method validation in a complex matrix. Although such good sensitivity was not enough to determine DOC circulating reference intervals in our healthy population, the inclusion of this hormone in the quantification method is however important for the diagnosis of some pathological conditions such as the 21-hydroxilase or 11-hydroxylase deficit that cause an increased concentration of this hormone. Moreover, using this high serum volume, also noise and interferences can increase; to avoid contaminations we developed the double purification: the SPE and the on-line purification with a perfusion column. The chromatographic gradient gave a good resolution for all the analytes, and especially for isobaric compounds, such as corticosterone and 11-deoxycortisol, DOC and 17OH-progesterone.

Once validated following the FDA guidelines[45] and verified by using certified products of the Reference Institute for Bioanalytics, the LC-MS/MS method has been compared with routine immunoassays employed in the Laboratorio Centralizzato of the Policlinico Sant'Orsola Malpighi in Bologna. The results of this study showed a good immunoassays performance concerning steroid hormones circulating at physiological high concentrations, such as cortisol, men testosterone and progesterone >1ng/ml, while problems due to the calibration of the immunoassays were highlighted for androstenedione and DHEA. Poor specificity of the immunoassays, extensively supported by literature data and documented in one of the previous

paragraphs[13-40], was found for women testosterone, 17OHprogesterone and progesterone <1ng/ml. In the first case, the poor specificity causes a low accuracy, as much as testosterone concentrations in some samples result in the pathologic range if analyzed with immunoassay and in the healthy range if analyzed with LC-MS/MS. On the other hand, the poor accuracy caused a systematic overestimation for 17OHprogesterone and a proportional overestimation for progesterone <1ng/ml. As told before, for the analytes corticosterone, 11deoxycortisol and DOC was not possible to perform the comparative study, since they are not currently measured at the Laboratorio Centralizzato.

Since the clear limits of the immunoassays were confirmed, we used the method to redefine the reference intervals in a healthy population composed of 356 subjects (179 adult males and 177 adult females). This thesis work, recently published (Annexes), has been one of the first work in which an LC-MS/MS multi-analytes quantification method of a wide steroids profile is used for the definition of reference intervals. Our data showed a strong androgens tendency to decrease proportionally with age in both men and pre-menopausal women. While testosterone and androstenedione are stable in post-menopausal women, DHEA tends to decrease in all the groups. In addition, post-menopausal women presented an age-dependent decrease also for glucocorticoids, both cortisol and 11-deoxycortisol.

In general our values of cortisol and corticosterone are lower than those reported in previous studies[101-104]. The explanation could be the saline infusion performed to our patients to avoid stress-related alterations in the glucocorticoids cascade. However further wide population screening are required to better establish steroids circulating reference intervals, grouping the population for decades.

Afterwards, steroids circulating concentrations were determined in an overweight population and an obese population. We compared then the results obtained in these two cohorts with a group of normal weight people extracted from our healthy population, matching the three subjects groups by age. Concerning the women, we found significantly lower cortisol levels in the obese group compared to overweight and normal weight women: these data could be apparently in contrast with data literature, since obesity presents an hyper-activation of the HPA axis that should lead an increased production of glucocorticoids. To better understand these data, a determination of the circadian rhythm of this hormone is necessary, since single-time cortisol measurements are inadequate to determine the activation status of the HPA axis[105]. Furthermore, also a measurement of cortisone, the inactive metabolite of the cortisol, can elucidate better the meaning of this controversial result.

Concerning men, we found highly significant lower levels of testosterone in the obese population, in agreement with what reported in literature from other authors. Indeed, these data concerning testosterone are not surprising: obesity in adult males is associated with hypogonadotropic

hypogonadism[106] and it is well known that infertility can be an obesity consequence, supported by low testosterone levels and an altered androgen-to-estrogens ratio.

In conclusion, the great technical approach provided by the LC-MS/MS for endocrine measurements of different hormones classes can be used to provide a rapid, simultaneous and reliable quantification indispensable for diagnoses and monitoring of a wide range of pathologies. The introduction of this technique in the clinical routine can contribute to more accurate diagnoses based on more precise laboratory data.

3

CIRCULATING ENDOCANNABINOIDS QUANTIFICATION IN OBESE PATIENTS BEFORE AND AFTER GASTRIC BYPASS SURGERY

The *endocannabinoid system* (ECS), principally consisting of *cannabinoid receptor type 1* (CB1), *cannabinoid receptor type 2* (CB2) and their endogenous ligands, the fatty acid derivatives *endocannabinoids* (ECs), is involved in a plethora of physiopathological processes, some of which are not fully understood[107]. Since the ECS is ubiquitously located, many key regulatory actions have been attributed to ECs, but several studies during the last twenty years have highlighted the prominent role of this system in the framework of food intake and energy balance[14]. ECs, in this context mainly acting through CB1 receptors[108], seem to have principally an anabolic function increasing food intake, promoting fat storage and decreasing energy expenditure, generally contributing to visceral fat accumulation in animals[14]. These ECs actions are exerted via both central[109] and peripheral[110] mechanisms. For instance, CB1 in the central nervous system (CNS) is highly expressed in hypothalamic nuclei involved in the control of energy balance and food intake and in neurons of the mesolimbic system involved in the regulating reward processes associated to food intake. Also in the periphery CB1 expression has been found in organs involved in the maintenance of the energy homeostasis, such as the white adipose tissue, the liver and the gastro-intestinal tract.

While in animals ECs functions have been extensively characterized[15-16], fewer studies in humans have been carried out. The evidence obtained from the ECS characterization in murine models led some years ago to develop a CB1 antagonist molecule as anti-obesity drug, which was called Rimonabant[14]. Despite encouraging results obtained from phase III clinical trials, Rimonabant was withdrawn from the market because of important psychiatric side effects such as depression and mood disorders. This disappointing clinical experience has shown how little we know about the ECS in humans.

To better understand the role of this system in the context of human obesity, less than ten years ago circulating levels of ECs started to be measured in human plasma using chromatography coupled with mass spectrometry. Nowadays, it is well known that an up-regulation of plasma ECs exists in obese subjects[17-19], despite the precise role of these circulating molecules is still unclear. Moreover, a correlation between ECs levels and anthropometric parameters related to obesity was found by several authors[19-20], as well as alterations of ECs circulating levels depending on the feeding status[22] or a diet-induced weight loss[21].

Based on the evidence found so far concerning higher circulating levels of plasma ECs in obese patients and the decrease of these levels after a weight loss[21], we decided to carry out a project aimed at evaluate ECs circulating levels in obese patients before and after a particular type of bariatric surgery intervention, the Roux-en-Y gastric bypass (RYGB), currently the most effective long-term anti-obesity therapy, achieving not only the best weight-loss percentage, but also inducing important metabolic improvements, including better control of type 2 diabetes[111-112]. This part of the thesis project was carried out at the Neurocentre Magendie INSERM U862 in Bordeaux.

This project consists of the evaluation of basal levels of circulating ECs at three stages of the study, before the surgery, one month after the surgery and six months after the surgery; the estimation of variations of plasma ECs values before and after the consumption of a controlled meal at the same three stages and the possible relationship between ECs changes and alterations of metabolic and anthropometric parameters resulting from the weight loss induced by the surgery. Our data could lead to better understand the role of circulating ECs in energy balance regulation and their role in possibly mediating weighloss and metabolic effects induced by the bariatric surgery.

Moreover, it was shown that 40% of obese non-diabetic men and 50% of obese diabetic men have decreased circulating concentrations of testosterone[113]. As explained in the former chapter, obesity is one of the chronic pathological conditions more frequently associated with hypogonadism, the decreased functionality of the gonads that leads to reduced circulating levels of sex steroids. The reasons underlying this deregulation are still poorly understood[114] and some data from clinical studies evidenced a recovery of normal plasma testosterone concentrations after a weight loss induced by RYGB[115-117]. In this context, we want to evaluate the recovery of normal androgens circulating concentrations, in particular testosterone and DHEA, in our obese patients undergoing RYGB. Moreover, since we measured fasting androgens before, one month after the surgery and six months after the surgery, we could analyze androgens concentrations in relation to different percentage of weight loss.

In addition, early evidence highlighted a connection between these two biological systems in animals: first, several constituents of the ECS are present on the HPG axis; second, ECs are involved in biological functions controlled by sex steroids, such as reproduction and sexual arousal[118]. Even if there are some discrepancies between different studies, several investigations carried out in 70s showed also that chronic marijuana smokers have decreased levels of testosterone[119-120]. As well as *Δ^9-tetrahydrocannabinol* (THC), AEA injections reduced testosterone levels in male wild-type mice but not in CB1 knock-out mice, suggesting that this effect is CB1 mediated[121]. Despite a consistent number of studies carried out in animals, the relationship between circulating levels of ECs and androgens in humans has been poorly investigated. Once assessed the alterations that may occur in plasma concentrations of both types of analyzed molecules in our obese patients, it will be possible to evaluate whether a correlation exists between circulating levels of ECs and androgens related to different obesity conditions. These data could provide a better understanding of the link between these two systems in humans.

I. The endocannabinoid system

Ia. Historical notes

The wide recreational, medical and religious use of the *Cannabis Sativa* has been documented since ancient times with medicinal benefits encompassing anesthetic, airway opening, anti-hypertensive as well as anti-emetic actions[122]. Despite this, the underlying physiological and molecular mechanisms were unknown. The first scientific turning point concerning the physiological characterization of the cannabis effects was in 1964 when Gaoni and Mechoulam first isolated and described the chemical structure of the terpenoid derivative Δ^9-THC[123], the main psychoactive component of marijuana.

In parallel with the discovery, Δ^9-THC and other hemp constituents were tested for psychotropic and motor effects in man and in animal models, mostly in mice, rats, rabbits, and dogs. Δ^9-THC proved to be the most effective among all phytocannabinoids being responsible for the vast majority of effects such as motor disturbances and catalepsy, corneal areflexia (in rabbits), scratching, euphoria and dysphoria, anxiety, drowsiness, altered time and audiovisual perceptions, panic attacks and impaired memory[124].

The underlying mechanisms for these effects were mostly believed to result from "non-specific" interactions between the lipophilic Δ^9-THC and the cell membranes, changing the fluidity and structure of the latter[122]. However this view of Δ^9-THC action is outdated by the discovery of Matsuda and co-workers in 1990 that cloned the CB1[125], a G protein-coupled receptor, which binds cannabinoids and whose activation is responsible for the Δ^9-THC effects. Three years later another cannabinoid receptor, the CB2, was discovered but its expression was found to be restricted mainly to immune tissues[126].

The presence of specific receptors for cannabinoids suggested the existence of endogenous ligands which could mimic Δ^9-THC effects: in 1992 Devane and colleagues described the chemical structure of the lipid molecule *N-arachidonoylethanolamine* also called *anandamide* (AEA), the first EC, isolated from brain and determined by mass spectrometry and nuclear magnetic resonance spectroscopy[127]. The name anandamide takes origin from the Sanskrit word "*ananda*", which means "bliss". In 1995 a second endogenous ligand for CB1 was discovered, the *2-arachidonyl glycerol* (2AG), which was isolated from the gut [128]. In the figure 3.1 the cannabinoid system time-line is represented.

Cannabinoid receptors, endocannabinoids and the enzymatic complexes for their synthesis and degradation represent together the endogenous signaling system, the ECS. In the last twenty years, it has become evident the ECS involvement in numerous mammalian physiological processes ranging from memory to stress response, from pain perception to reproduction: this evidence triggered an increasing number of studies in many medical research fields exploring all the functions and alterations of the ECS in

physiological and pathological conditions. Amongst all the regulatory functions attributed to ECS, its involvement in the food intake and energy balance mechanisms seems to be crucial[11-14]. ECs regulate many essential mechanisms in this context, for instance hunger, nutrients metabolism, regulation of body weight and rewarding proprieties of food[14]. The ECS system seems to be one of the principal candidates for the future therapeutic strategies against obesity. However, despite the increasing number of studies carried out so far aimed to discover the basic mechanisms underlying the actions of ECs in the human metabolism, further studies are required in order to further expand our knowledge in the field.

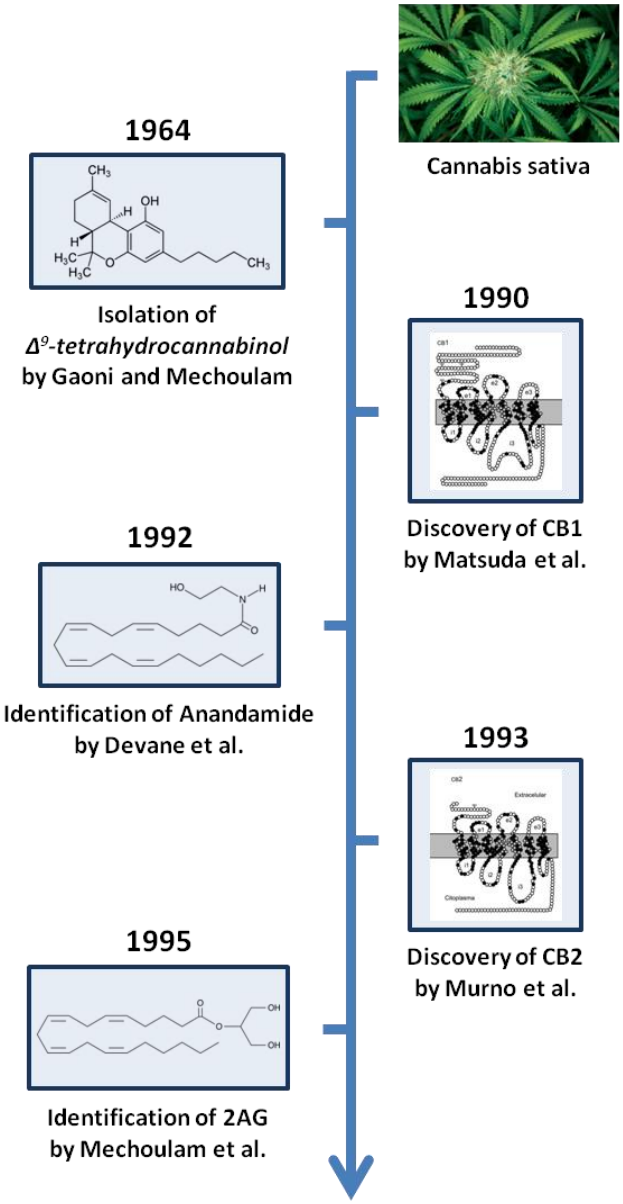


Figure 3.1: Cannabinoid system time-line.

Ib. Cannabinoid receptors

CB1 and CB2 are G protein-coupled receptors, mainly of the $G_{i/o}$ inhibitory subtype[107]. They are constituted by seven transmembrane domains connected by three extracellular and three intracellular loops and two hydrophilic tails: an extracellular N-terminal tail (shorter in the type 2 receptor) and an intracellular C-terminal tail that interact with the G-protein. The receptor activation leads to the activation of the $G_{i/o}$ protein, inhibiting *adenylate cyclase* with consequent decrease of cytosolic levels of the second messenger *cyclic adenosine monophosphate* (cAMP). In the CNS the activation of these receptors leads to the inhibition of the transmembrane ionic conductance, activating K^+_A channels and inhibiting N and P/Q type Ca^+ channels. Furthermore, their activation can stimulate intracellular kinases such as the *focal adhesion kinase* (FAK), *phosphatidyl-inositol 3-kinases* (PI3-K), ERK kinases and the p38 MAP kinase**.** The kinases activation can mediate the CB1-induced expression of the *immediate early genes* (IEG), such as the transcription factors c-fos, c-jun, zif268 and the *brain-derived neurotrophic factor* (BDNF)[14]. These pathways are summarized in figure 3.2 (adapted from *Pagotto U. et al.* 2006[14]).

The distribution of the cannabinoid receptors has been extensively studied using some different approaches, such as the autoradiography in brain sections[129], in situ hybridization[130] and immunohistochemistry[131]. According to receptor expression studies, CB1 receptor is the most abundant G-coupled receptor in the mammalian brain and it's highly expressed in hippocampus, neocortex, amigdala, in some region of the basal ganglia, thalamic and hypothalamic nuclei, cerebellar cortex and brainstem nuclei and the olfactory bulb[14]. Furthermore, last year Benard and co-workers found CB1 expression also in membranes of neuronal mitochondria, evidencing its direct involvement in cellular respiration and energy production[132].Originally, CB1 was defined as the "brain type" receptor because of its high expression in the CNS; however, additional studies concerning its expression have confirmed also the presence of this receptor in many peripheral organs such as for instance the thyroid, the adrenal glands and the testicles. Importantly CB1 is expressed in peripheral tissue involved in the energy metabolism: almost ten years ago, CB1 was found in human and animal adipocytes[15-133] and later expression of this receptor was found also in the liver[134], pancreas[135] and skeletal muscle[14]. CB1 is also present in the gastro-intestinal tract (GI)[136], where it modulates many important functions, such as gastric emptying, intestinal motility and secretions[137]. In addition, CB1 is expressed in vagal nerve terminal of the GI involved in the modulation of food intake[136]. The CB2 receptors, conversely, are expressed almost only in immune cells[138].

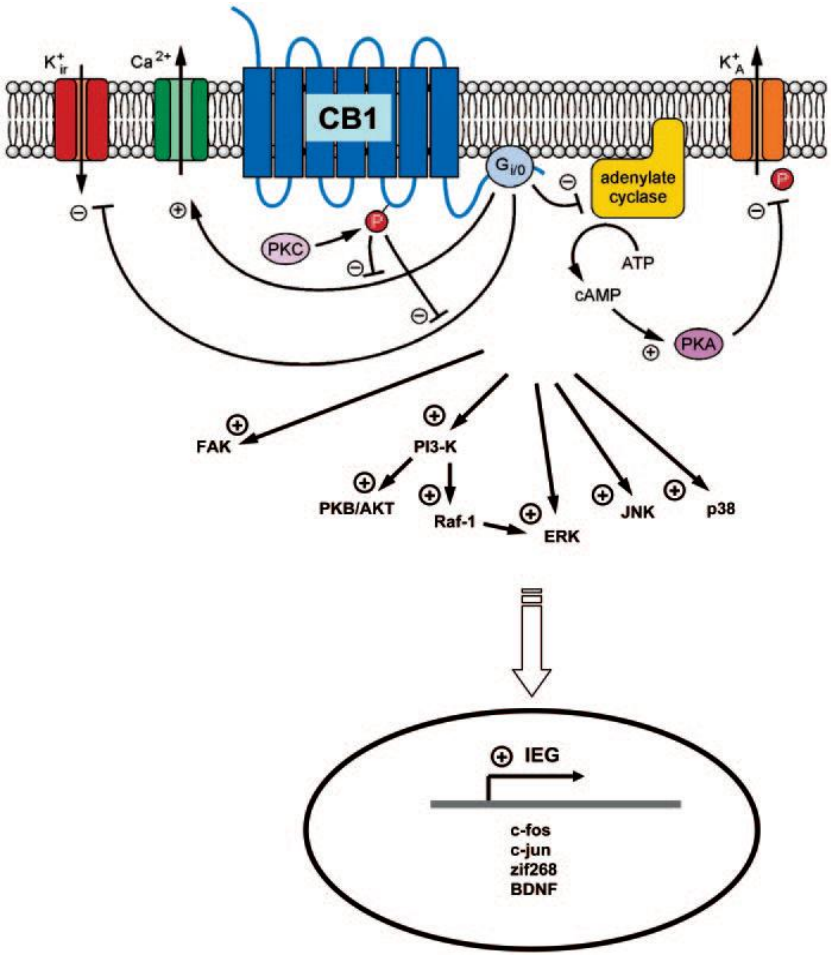


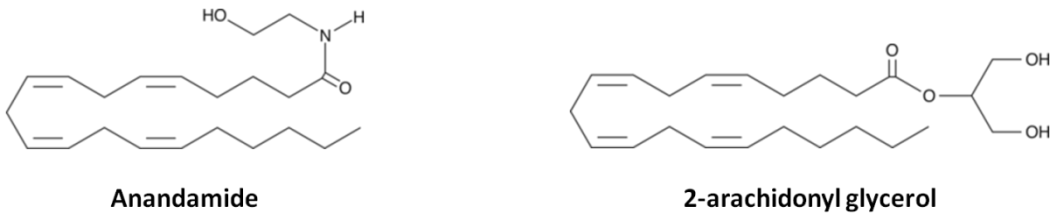
Figure 3.2: CB1 intracellular signalling.

Ic. Endocannabinoids

ECs are lipids, polyunsaturated fatty acids derivatives of arachidonic acid. Five ECs have been described so far, but only two of them are present at appreciable physiological concentrations and bind CB1 with considerable affinity: AEA and 2AG (figure 3.3). For this reason, these two ECs are the more studied and best characterized.

Given their lipophilic nature, ECs are not stored after their production, but released as soon after their synthesis exerting their biological functions on neighboring cells or on the same cell which produced them, following an "*on demand*" production mechanism[139]. Therefore, since they are local mediators, it has been hypothesized that ECs found in plasma represents a "spill-over" from tissues rather than a biologically active fraction[140].

Moreover, it has been shown that in the brain ECs behave as *retrograde messengers*: they are first synthesized in the postsynaptic cell and then they activate the CB1 receptor expressed on the presynaptic cell[139].



Anandamide **2-arachidonyl glycerol**

Figure 3.3: Anandamide and 2AG chemical structure.

1. AEA

As mentioned before, AEA was the first endogenous molecule binding cannabinoid receptors identified. It binds CB1 receptor with appreciable affinity and, when injected in the rat brain, induces the same effects of THC[127]. The AEA biosynthesis occurs on demand and it is a two steps process: first, the AEA precursor *N-arachidonoyl- phosphatidylethanolamine* (NAPE) is synthesized by a reaction catalyzed by the enzyme *N-acyltransferase* (NAT) consisting in the intermolecular passage of an arachidonic acid group from the Sn-1 position of phosphatidylcholine to the head group of phosphatidylethanolamine. The second reaction is a stimulus-dependent cleavage of the NAPE mediated by a *phospholipase D*[141] (NAPE-PLD) producing AEA and phosphatidic acid. The reactions of the AEA biosynthesis are represented in figure 3.4 (adapted from *Piomelli D.*, 2003[142]). NAPE-PLD is widely distributed in mammalian tissues; expression studies revealed the high content of this enzyme in mice brain, kidney and testicles[141]. A moderate expression of NAPE-PLD was also found in organs directly involved in energy metabolism, such as the large intestine and the skeletal muscle[141].

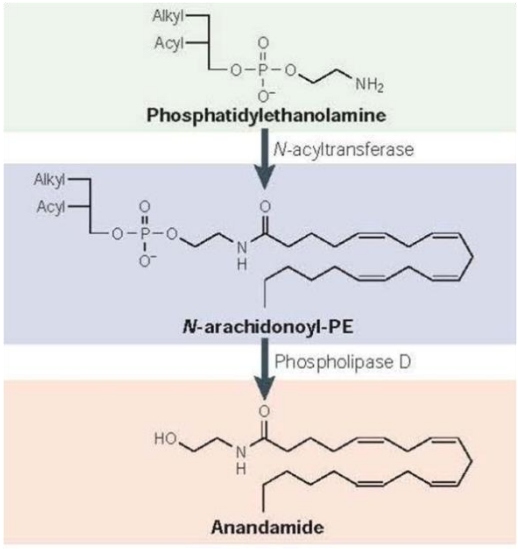


Figure 3.4: AEA biosynthesis.

Two kinds of second messengers control the NAT activity: Ca^{2+} and cAMP[142]. Ca^{2+} is directly required for NAT activity, since NAT is inactive without it. cAMP activ NAT indirectly through the NAT phosphorylation by a protein kinase A-dependent mechanism. It was shown that AEA is produced and released from cultured rats brain neurons in a calcium ion-dependent manner: incubating the cultured neurons with [^{3}H]-ethanolamine, Ca^{2+} ionophores, such as the ionomycin, stimulate the [^{3}H]-AEA accumulation[143]. There is

also some evidence of regulation of AEA biosynthesis through G protein-coupled receptor: Giuffrida and colleagues found an increase of AEA concentration after administration of quinpirole, a D2 like dopamine receptor agonist, in rat dorsal striatum that can be prevented by raclopride, a D2 like receptor antagonist[144].

AEA was isolated for the first time from brain extracts[127]; several studies suggested that AEA content varies across different brain regions, but quantitative analyses are not always consistent[145]. A high AEA amount has been found in hippocampus and striatum; appreciable quantities in midbrain, cortex, hypothalamus and cerebellum[145]. In the periphery, a particularly important production of AEA has been found in tissues involved in the control of energy homeostasis, such as the gut, the liver, the WAT, the skeletal muscles and the pancreas[140].

AEA action is regulated by a two steps process: after its biological actions, it is first internalized into the cell and then hydrolyzed by the *fatty acid amide hydrolase* (FAAH)[146]. FAAH is an intracellular membrane-bound serine hydrolase with an N-terminal transmembrane domain, which is the principal catabolic enzyme of AEA, generating arachidonic acid and ethanolamine. This enzyme is widely expressed in the rat brain with a main presence in principal neurons of the cortex, the hippocampus, the cerebellum and the olfactory bulb[147], but moderate FAAH expression was found in almost all the major brain areas[148]. Concerning the periphery, FAAH expression was found in different organs, some of which involved in the energy balance regulation such as the liver[148], the pancreatic beta-cells and the white adipocytes[17].

Furthermore, AEA neuronal breakdown is mainly a post-synaptic process[142]. The AEA removal from the extracellular space and the transport of it into the cytosol is still unclear; an interesting mechanism was proposed by Piomelli and co-workers, finding a *FAAH like anandamide transporter* (FLAT) which lacks the catabolic action typical of FAAH but can bind anandamide with lower affinity, transporting it into cells[149].

2. 2AG

2AG is a monoacylglycerol, omega6 fatty arachidonic acid and glycerol ester. Two possible synthesis pathways were described. First, it can derive from the conversion of phosphatidylinositol in *1,2-diacylglycerol* (DAG) mediated by the enzyme *phospholipase C* (PLC) and the consequent hydrolysis of DAG in 2AG trough the enzyme *diacylglycerol-lipase* (DAGL)[150]. An abundant expression of DAGL was found in the rodent hypothalamus, while a mild expression of this enzyme was found in the majority of extra-hypothalamic brain areas[150]. In periphery, the most copious presence of DAGL was found in human pancreas, however the presence of this enzyme was observed in many other human peripheral tissues, such as for instance the adipose tissue or the liver[150].

The second pathway proposed is the synthesis of a 2-arachydonoyl-lysophospholipid mediated by the enzyme phospholipase A1 (PLA1); the 2-arachydonoyl-lysophospholipid is then hydrolyzed by a lyso-PLC[142]. The synthesis pathways of 2AG are summarized in figure 3.5 (adapted from *Piomelli D.*, 2003[142]). The 2AG is the most abundant endocannabinoid in the brain: its concentration is about 170 fold higher than anandamide[151].

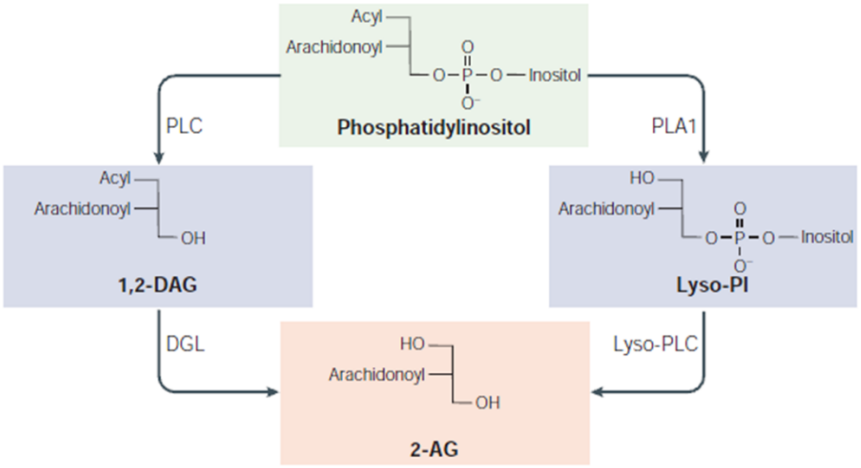


Figure 3.5: 2AG biosynthesis.

2AG was isolated for the first time in the gut[128], a tissue directly involved in the energy balance. Similarly to AEA, 2AG production seems to be important also in the WAT, the liver and the skeletal muscle[140]. 2AG

concentrations in brain areas are not different as for AEA[145]: this EC has been found in considerable amount in almost all the major brain regions, in particular the hypothalamus, the hippocampus and the midbrain[145].

The principal enzyme responsible for the 2AG degradation is the *monoacylglycerol lipase* (MAGL) a membrane-bound serine hydrolase that catalyzes the 2AG break-down generating fatty acid and glycerol. Like FAAH, also MAGL is widely diffused in many brain areas, with higher expression in the cerebellum, the cortex, and the hippocampus, but while the FAAH distribution is principally post-synaptic, MAGL was found mainly presynaptically[152]. The 2AG re-uptake mechanism is still unclear.

II. Endocannabinoids and obesity

IIa. Animal studies and early evidences

Since ancient times it was known that the consumption of cannabis in different forms was able to stimulate appetite, particularly for palatable foods[153]. The identification of THC as main psychoactive component of marijuana allowed investigating the appetite stimulatory effect of this molecule. Indeed, several animal studies demonstrated the ability of THC to promote appetite, although significant effects were shown only with low doses[154]. After the discovery of cannabinoids receptors and the introduction of selective antagonists, the increased appetite induced by THC was clearly linked to the activation of the CB1 receptor, since its effect was inhibited by the CB1 selective antagonist SR141617 (Rimonabant) but not by the CB2 selective antagonist SR144528[155]. After the identification of ECs as endogenous ligands of the CB1 receptor, it was demonstrated that low AEA doses, injected systemically[156] or in the ventro-medial nucleus of hypothalamus[157], stimulate the appetite. A similar increase of food intake was produced also injecting 2AG systemically or in the nucleus accumbens[158]. Moreover, orexigenic sites of action of ECs in the hypothalamus and limbic forebrain suggested their involvement in both homeostatic and reward-related control of appetite[159].

The investigations carried out using CB1 antagonists have provided a direct support to the hypothesis by which ECs have a regulatory role on food intake. AM251, a CB1 antagonist, reduced appetite in rats fed ad libitum[160]. However, the most important evidence of the ECS involvement in the control of food intake was provided by the development of knock-out mice lacking the CB1 receptor. Indeed, knock-out mice eat less compared to wild-type littermates, and their food intake cannot be further modulated by Rimonabant, while wild-type mice treated with this CB1 antagonist showed a decreased food intake[108]. In addition, CB1 knock-out mice are resistant to the orexigenic action of neuropeptide Y (NPY) as well as wild-type mice treated with Rimonabant[161]. Recently, Bellocchio et al. demonstrated an opposite role of CB1 in regulating food intake in the brain: while modulation of glutamatergic transmission by CB1 exerts the well-known cannabinoid-induced orexigenic effect, CB1 expressed on GABAergic neurons mediate a surprising hypophagic effect, suggesting a central opposite regulation of food intake by the ECS[162]. In the interesting work carried out in 2001 by Di Marzo and colleagues, the authors showed an inverse relationship between levels of leptin , an anorexigenic hormone produced primarily by the adipose tissue, and hypothalamic ECs levels. In particular, they showed an increase in hypothalamic ECs levels linked to a defective leptin signaling in obese *db/db* and *ob/ob* mice, while acute leptin intravenous injections were able to decrease hypothalamic ECs[108].

In a study carried out in 2003, Cota and co-workers described in detail the phenotype of CB1 knock-out mice showing hypophagia and reduced body weight and fat mass compared to CB1 wild-type littermates, and evidencing the crucial role of the ECS in the energy homeostasis balance[15]. Moreover, authors proposed for the first time a food intake-independent mechanism contributing to the leaner phenotype of CB1 knock-out mice, suggesting that the reduced body fat in these mice could result from both central and peripheral mechanisms, such as hypothalamic alterations and deregulations of the adipocytes functions, since they first reported a role for CB1 receptors in the modulation of the adypocite metabolism[15].

Consequently, it was shown that CB1 knock-out mice were resistant to metabolic modifications induced by chronic exposure to a high-fat diet, such as high levels of circulating triglycerides and elevated plasma concentrations of leptin and insulin, a clue of leptin and insulin resistance[16-163]. Interestingly, changes of AEA concentrations were also found in the small intestine of mice related to fasting and re-feeding conditions[164]. AEA levels were higher in duodenum and liver following food deprivation and decreased after food intake, suggesting a possible peripheral mechanism involving ECs and food intake[164].

IIb. Human studies: circulating endocannabinoids in obesity

The studies carried out in animals have highlighted the important role of the ECS in the modulation of food intake and in the development of obesity. Despite the fact that the ECS is very well characterized in animal models, experimental evidence about its physiopathological role in humans is still lacking.

The detection of ECs in human plasma could contribute to better understand the relationship between ECs and human obesity. Although some ECs functions have been very well characterized, the role of the circulating ECs is still unclear. It has been found an up-regulation of circulating ECs in obese patients, and in some studies also a correlation between their levels and several parameters altered by obesity, such as waist circumference and BMI[19-20]. In 2005 it was first published the up-regulation of AEA circulating levels (but not 2AG) in obese women presenting binge eating disorders (BED) compared to normo-weight women[165] while in another study of the same year, both AEA and 2AG plasma levels resulted higher in obese post-menopausal women compared to lean women[133]. In the second study it was further found a positive correlation between AEA and BMI, 2AG and waist circumference. In 2006 Matias and co-workers found increased levels of both AEA and 2AG also in overweight diabetic subjects[17]. Studies about circulating ECs evidenced also the existence of a relationship between their plasma levels, insulin levels and the development of insulin resistance. In 2006 Bluher and colleagues found that 2AG circulating levels positively correlated with fasting plasma insulin concentrations and negatively correlated with glucose infusion rate during clamp, suggesting a direct relation between 2AG levels and glucose metabolism[19]. In 2009 it was proposed that insulin is as negative regulator of circulating levels of ECs: authors found an AEA decrease induced by glucose oral load inversely correlated to fasting levels of insulin and glucose, suggesting that the ability of insulin to negatively regulate plasma ECs is lost in the presence of insulin resistance[166].

i. Endocannabinoids and weight loss

Few studies have been carried out so far about modifications in circulating ECs levels after weight loss. In the first study in obese post-menopausal women a 5% of total body weight loss obtained in 13-15 weeks [133]. Moreover, in this study also CB1 and FAAH mRNA levels in adipose tissues were evaluated, and neither resulted significantly influenced by the weight loss[133]. In another study, authors investigated changes in circulating ECs in twenty randomized non-diabetic obese patients treated with *sibutramine*, an inhibitor of neurotransmitters reuptake used as anorexigenic drug, withdrawn from the market in 2010. Patients were first treated with 15mg/day of the drug or placebo for 5 days (acute treatment) and then they were treated with open-label 15mg/day of sibutramine (chronic treatment). Neither the acute treatment nor the chronic treatment caused significant changes in circulating AEA and 2AG after a body weight loss of 6.0 ± 0.8 kg obtained after three months[167]. On the contrary, in another study in 2009 significant changes were observed in plasma ECs after a weight loss induced by life style modifications[21]. 49 asymptomatic non-diabetic obese men followed a life-style modification program including a diet and physical activity exercise during 1 year. At the end of this period, subjects presented reduced body weight (-6.4kg), BMI (-2.1kg/m^2) and waist circumference (-8.0cm) and also ameliorated parameters implicated in obesity co-morbidities, such as triglycerides (−0.74 mmol/l), HDL-cholesterol (+0.13 mmol/l) and fasting insulin levels (−51.9 pmol/l). Both AEA and 2AG levels were decreased after the weight loss (AEA: -0.54 pmol/l, $p<0.005$; 2AG: -0.98 pmol/l, $p<0.005$)[21].

Since different results were achieved by different studies, it is still unclear whether the weight loss can directly lead to variations of circulating ECs levels. Noteworthy, experimental parameters and procedures of the three studies discussed above were not the same, such as weight loss strategies, patients characteristics and ECs measurement time-point: therefore, is likely that these experimental discrepancies are the reasons behind the inconsistency of the obtained results. In particular, it is possible that in the first two studies some weeks of pharmacologic and life-style treatment were insufficient to achieve significant changes. The latter study was carried out after one year, during which patients achieved an adequate weight loss that led to physiological ECs changes.

Summarizing, in obese patients circulating ECs levels are higher compared to normo-weight subjects; and after weight loss obtained with life-style modifications plasma ECs tend to decrease. Studies carried on so far clarifying the meaning and alteration mechanisms of circulating ECs are still insufficient; starting from what has been found so far, further data are required to elucidate the role played by these molecules in the framework of the human obesity.

ii. Endocannabinoids and food intake

Several studies in animals showed changes of ECs levels in tissues such as the hypothalamus and limbic forebrain in the CNS[168] or in peripheral tissue, such as the duodenum[164] related to the animal feeding status. In 2006, comparable changes of ECs were found also in human plasma by Matias and co-workers: they described a significant post-prandial decrease of AEA in plasma after the consumption of a meal with high content of fat in normo-weight subjects[17].

These results were further confirmed in 2012, when a precise kinetics of plasma AEA levels related to the food intake was described. In normo-weight subjects, AEA presented an increase in pre-prandial blood concentrations and a post-prandial decrease of almost the 40%, displaying a circulating profile of a hunger signal. Obese patients, while retaining the pre-prandial AEA peak, have a blunted post-prandial decrease in plasma AEA (approximately 10% of post-prandial change) after the consumption of a calorically balanced meal (figure 3.6 A-B modified from *Gatta-Cherifi et al.*, 2012)[22]. Concerning 2AG, it was confirmed what was previously found in 2006: its circulating levels are not modified by the feeding status, suggesting a different role for 2AG in the context of food intake (figure 3.6 C).

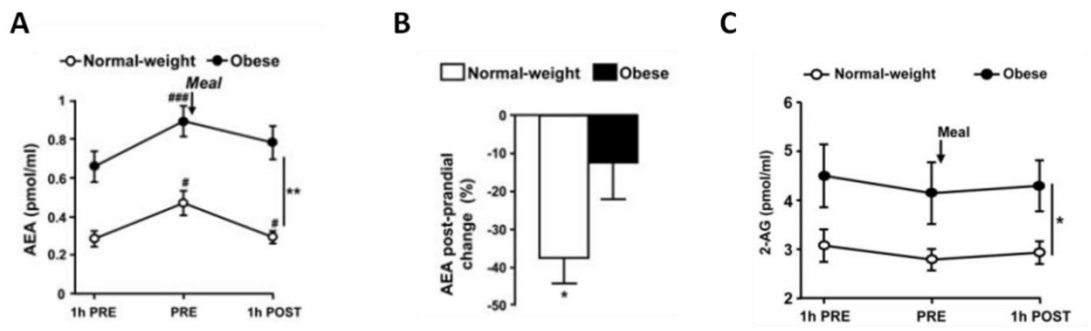


Figure 3.6: AEA circulating concentrations changes related to the feeding status in normal-weight and obese people (**A**). Circulating AEA post-prandial percentage of change in normal-weight and obese people (**B**). 2AG circulating concentrations changes related to the feeding status in normal weight and obese people (**C**).

Similarly, the authors also reported a meal-related kinetics of the anorexigenic peptide YY (PYY) that showed in normo-weight subjects opposite changes compared to AEA, significantly increasing after the meal consumption. While PYY levels did not change in obese subjects in response to the meal (figure 3.7 A-B modified from *Gatta-Cherifi et al.*, 2012[22]), suggesting an obesity-dependent deregulation of the levels of this hormone, as previously reported[169].

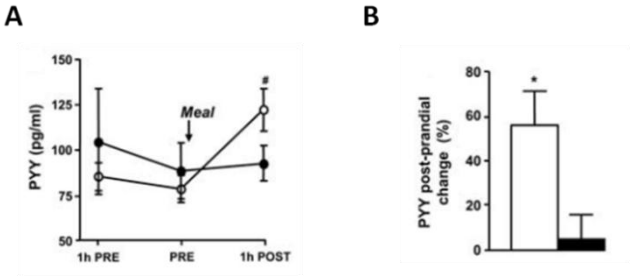
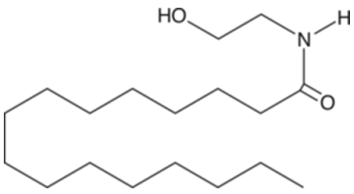


Figure 3.7: PYY circulating concentrations changes related to the feeding status in normal-weight and obese subjects (**A**). Circulating PYY post-prandial percentage of change in normal-weight and obese subjects (**B**).

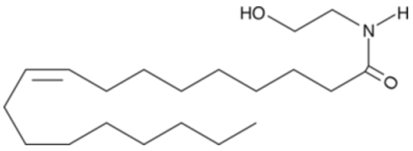
At this stage, it is still not clear where AEA changes originate from. A natural candidate could be the gastro-intestinal tract: the presence of the ECS has been extensively documented in the gastrointestinal tract, where CB1 and CB2 receptors have been found, and ECs and enzymes involved in both their production and degradation have been quantified. Furthermore, as mentioned before, ECs in gastrointestinal tract tissues are increased during fasting and a decreased after refeeding in rodents[136-164].

IIc. The emerging role of endocannabinoids related compounds

In addition to the well characterized AEA, other N-acylethanolamines (NAEs) could be involved in food intake and obesity mechanisms: *oleoyl-ethanolamine* (OEA), amide of oleic acid and ethanolamine, and *palmitoyl-ethanolamine* (PEA), amide of palmitic acid and ethanolamine. Since they belong to the same chemical class and share the same biosynthetic and degradative pathway, PEA and OEA are structurally very similar to AEA (figure 3.8).



Palmit-oylethanolamide



Oleoyl-ethanolamide

Figure 3.8: PEA and OEA chemical structure.

Despite these similarities, PEA and OEA are inactive on cannabinoid receptors[170]. Both these compounds exert their biological proprieties binding the nuclear receptor *peroxisome proliferator-activated receptor alpha* (PPAR-α)[170]. Some studies suggested a link between the action of these two compounds and AEA: OEA and especially PEA can be an alternative substrate for FAAH, the AEA catabolic enzyme, reducing in this way the degradation rate of AEA and enhancing its action[171]. For this reason, PEA and OEA are defined as "entourage" compounds. Giving their structural similarities and related actions with AEA, an involvement of these molecules in the energy homeostasis and food intake has been supposed[172].

i. Animal studies

Given the actions exerted by AEA and 2AG in energy balance and obesity development in animals, studies were carried out also to assess whether OEA and PEA were involved in the same mechanisms.

In 2001, Rodriguez de Fonseca and co-workers found decreased concentrations of OEA in the small intestine in rats after food deprivation. OEA administration caused reduction of food intake, suggesting an anorexic effect of this molecule, mostly at peripheral levels, while PEA was significantly less potent than OEA in exerting this action[173]. Two years later, it was demonstrated by the same group that the anorexic action and the reduction of body weight gain induced by OEA required PPAR-α receptors, since this effect is lost in PPAR- α knock-out mice[170]. Furthermore, it was shown that OEA injection is able to enhance lipolysis in wild-type mice but not in PPAR-α knock-out mice[174]. In a recent study, Schwartz and colleagues found increased OEA mobilization after fat injection in the duodenum, but not after proteins or carbohydrates infusion. They hypothesized a specific link between ingestion of fats and satiety induced by intestinal OEA mobilization[175].

Unlike OEA, PEA functions concerning food intake and obesity pathophysiology are less characterized. PEA seems to exert its main biological role as anti-inflammatory molecule. This peculiar effect was shown to be carried out through PPAR-α receptors, since is lacking in PPAR-α knock-out mice[176]. Likely, PEA could have a role in obesity-induced inflammation.

ii. Human studies

As for AEA and 2AG, the role of circulating PEA and OEA in human obesity is not clear. Few studies were carried out so far measuring these ethanolamines in the plasma of obese subjects. In one of the first human studies investigating expressly these molecules[172], PEA and OEA were measured in visceral fat and subcutaneous fat of two groups of subjects: obese patients and normo-weight controls. No significant differences between the two groups were found in visceral fat content of PEA and OEA; on the contrary, significantly lower PEA concentrations were found in subcutaneous fat of obese subjects compared to visceral fat[172]. In the same study, fasting PEA and OEA were quantified in blood of diabetic over-weight patients compared to healthy volunteers finding higher concentrations of both the NAEs in diabetic over-weight patients. Furthermore, they found a decrease of post-prandial circulating PEA and OEA in healthy volunteers after a meal with high-fat content[172]. In 2010, increased OEA concentrations were found in 20 subjects affected by sleep apnea compared with over-weight healthy controls; also a correlation between OEA circulating levels and BMI was found in the sleep apnea group[177]. Last year, Matias et al. measured PEA

and OEA, in association with endocannabinoids AEA and 2AG, in saliva and they found elevated concentrations of the four analytes in obese subjects compared to normal-weight people[178].

Further knowledge is therefore required to better understand the physiological role of these circulating compounds in relation to obesity and food intake, measuring them in different conditions.

III. Anti-obesity approaches

Nowadays, in case of severe obesity (BMI>30 kg/m^2) when life-style modifications are not sufficiently effective, two kinds of therapeutic strategies are currently available, drug therapies and bariatric surgery.

IIIa. Drug therapies

Three drugs developed so far have proved to be successful in decreasing significantly body weight: Sibutramine, Orlistat and Rimonabant, a CB1 antagonist.

Sibutramine is an inhibitor of neurotransmitters serotonin and norepinephrine reuptake, which has an effect on both food intake and energy expenditure[179]. In 1997, this drug was approved by FDA and in 1999 its use was authorized in the European Union (EU)[180]. Unfortunately, data from the Sibutramine Cardiovascular Outcomes Trial (SCOUT)[181] proved an increased risk of myocardial infarction and stroke, and consequently, this drug was withdrawn from the market in 2010, first in EU and then in USA and other countries[180].

Orlistat is the first anti-obesity drug that is not acting as an appetite-suppressant; indeed, it causes an inhibition of pancreatic and gastric lipases, by which 30% of ingested dietary fat remains undigested and unabsorbed leading in particular to ameliorations of lipid profile parameters[182]. This drug was approved by FDA in 1999, and it is still on the market in approximately 100 countries all over the world, despite its gastro-intestinal side effects[180]. Noteworthy, Orlistat is less effective than other anti-obesity drugs; however, it is the only one currently available on the market [180].

Given the scarce availability of anti-obesity drugs, based on the studies carried out on animals, in 1994 the pharmaceutical company Sanofi-Aventis developed a CB1 antagonist, the Rimonabant (commercialized as *Acomplia*®)[183]. Despite it was at the beginning tested as a smoking cessation agent[184], evidence about the efficacy of this CB1 antagonist in animal studies led Sanofi-Aventis to test it as pharmacological strategy against obesity in humans at the beginning of 2000s.

Several studies evaluated the efficacy of Rimonabant in the treatment of obesity: in August 2001 a wide phase III study called[185] the *RIO program* (Rimonabant in Obesity) started, including more than 6600 patients all over the world. The study was constituted by 4 controlled randomized trials: *RIO-Europe*, *RIO-Lipids*, *RIO-North America* and *RIO-Diabetes*. RIO-Lipids and RIO-Diabetes were more focused on the amelioration of specific obesity co-morbidities (hyper-lipidemia and diabetes mellitus type II, respectively), while RIO-Europe and RIO-North America recruited obese and over-weight patients with or without co-morbidities[14]. All the patients involved (1507 for RIO-Europe, 1036 for RIO-Lipids, 3045 for RIO-North

America and 1045 for RIO-Diabetes) were treated for two years with Rimonabant 5 mg/day or 20 mg/day or placebo. Furthermore, subjects followed also a life-style modification program and a hypo-caloric diet together with the drug therapy. The primary end-point of RIO-North America was the evaluation of the body weight loss during the first year and the prevention of weight regain after a further patients randomization at the beginning of the second year of the study. On the other hand, the main end-point of RIO-Europe was the evaluation of body weight loss[14]. Similar results were obtained at the end of each trial: data from RIO-Lipids showed a weight loss of more than 10% of the initial body weight in the 44.3% of patients included in the Rimonabant 20mg group (p:0.001 vs placebo), 16.3% of patients included in the Rimonabant 5mg group and 10.3% of placebo group patients. The Rimonabant 20mg group showed also substantial and significant reduction of waist circumference, circulating triglycerides, C-reactive protein and a significant increase of HDL cholesterol compared to the placebo group[186]. Moreover, during this trial Rimonabant resulted generally well tolerated, reporting the main side-effect regarding the gastro-intestinal tract (nausea) or upper respiratory tract infections[186]. Similarly, data from RIO-Europe showed a weight loss significantly greater in patients treated with Rimonabant 20mg (-6.6kg) and Rimonabant 5mg (-3.4kg) compared to patients treated with placebo (-1.8kg) after one year. Also the improvement of waist circumference, HDL-cholesterol, triglycerides and insulin resistance was greater in the Rimonabant 20mg group. Transient and mild side effects were reported by a similar percentage of patients in all the groups[187-188]. Thanks to these positive results obtained by the RIO program, in June 2006 the European Medicines Agency (EMEA) approved the sale of Acomplia® for the treatment of obesity and related co-morbidities in Europe for patients with a BMI ≥ 30 or BMI > 27 with severe co-morbidities, such as diabetes type II or dyslipidemia. However, the side effects induced by Rimonabant therapy were higher than expected: patients under therapy described side effects on their mood such as depressive and anxiety symptoms. Thus, in the 2009 Acomplia ® was withdrawn from the market[189]. The withdrawal of this compound from the market also highlighted the necessity to improve the knowledge about the role of the ECS in the human physiology to better define future therapeutic strategies.

Currently new anti-obesity drugs are under investigation, some of them based on gut hormones-like molecules[180]. Indeed, the development of new anti-obesity drugs possibly having mild central and peripheral side effects is strongly needed in order to help tackle the obesity epidemic.

IIIb. Bariatric surgery: Roux-en-Y gastric bypass

Currently, the most effective long-term treatment for severe obesity is bariatric surgery, a variety of surgical procedures aimed at the obtainment of weight loss, mostly reducing the stomach size, to reach satiety more quickly, or bypassing an intestine part, reducing in this way the nutrients absorption. Different bariatric surgeries were developed and proved to be the best long-term treatment for obesity, successfully reducing body weight and obesity related co-morbidities. Among them, RYGB is the most performed (approximately 90% of bariatric surgeries[23]), since it achieves the best weight loss results and several metabolic ameliorations. This surgery provides a substantial and durable weight loss: 37% of total body weight after 1 year and the regain of the 7% of the body weight loss after 14 years. Furthermore, another interesting aspect of this bariatric surgery is the high percentage of patients with complete type II diabetes (T2DM) remission: approximately 80%[111].

i. Surgical procedure

RYGB is a malabsorptive and restrictive surgical procedure described for the first time in the early 1990s. It is performed creating a stomach restriction ("pouch") and an intestine bypass: the stomach capacity is reduced to 30 ml[111] allowing the patient to feel quickly sated with a less amount of food, while the intestine bypass reduces the nutrients absorption bypassing the duodenum and the jejunum. The intestine is rearranged with the typical "Y" shape in the new anatomical configuration and in this way the nutrients are directly carried to the distal gut. The operation is represented in figure 3.9.

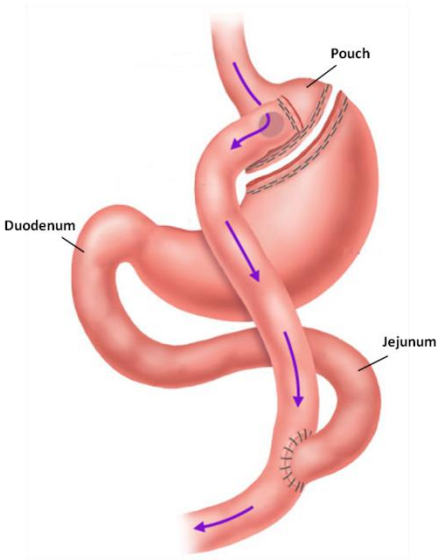


Figure 3.9: RYGB surgery. The stomach restriction leads to the formation of the "pouch". Bypassed intestinal parts are the duodenum and jejunum. Arrows indicate the ingested food route determined by the new anatomy.

ii. Roux-en-Y gastric bypass anti-diabetic effect

Several clinical studies have described a direct anti-diabetic effect of the RYGB. The first study was carried out in 1995: in 82.9% of patients with T2DM and 98.7% of patients with glucose impairment undergone this surgery ameliorations were observed for all the parameter relative to the glucose metabolism[111]. In 2005 another study was performed monitoring fasting glucose and glycated hemoglobin (HbA1C) plasma levels before, 6 months after and 12 months after the RYGB intervention. Significant decrease of both the analyzed parameters was observed one year after, with T2DM resolution in 74% of 177 included patients[112]. The high percentage of complete remission of T2DM after RYGB was further confirmed by other studies verifying also the anti-diabetic efficiency of the RYGB compared to the classic weight loss strategy: the diet[190]. This year a study was published in which the anti-diabetic efficacy of the RYGB was compared with sleeve gastrectomy and medical therapies, such as incretin analogues combined with a diet. All these three strategies achieved a better glycemic control after one year, but the RYGB showed the highest percentage of improvements: 12% of the patients with medical therapy, 37% with sleeve gastrectomy and 42% of patients with RYGB reached the first study end-point, the decrease of the HbA1C level of 6%[191]. Despite the number of published works about the relationship between this surgery and the diabetes remission, the precise physiological mechanism underlying this effect is still unclear. Noteworthy, it is now well accepted that the T2DM remission obtained after the RYGB is weight loss independent: firstly because 30% of patients present normal glucose levels approximately 3 days after the surgery[192-193] before the weight loss occurs; secondly because this anti-diabetic effect is not obtained with other types of bariatric surgery, although providing the same amount of weight loss[194].

Several hypotheses possibly explaining the specific RYGB anti-diabetic effect have been proposed. In 2002, Cummings and colleagues suggested the ghrelin hypothesis. Ghrelin is an orexigenic peptide produced mainly by the stomach and duodenum, both organs altered after RYGB. The authors measured circulating ghrelin in post-RYGB patients, finding extremely low levels of this hormone[195]. Their findings were due to the specific anatomical changes provided by the RYGB. The compromised ghrelin secretion after the operation can improve the glucose tolerance, since ghrelin inhibits insulin secretion, hepatic insulin signaling and secretion of adiponectin, a peptide involved in the glucose regulation and fatty acid break-down[196].

Another interesting hypothesis was suggested by Morinigo et al. in 2006. They found increased levels of glucagon-like peptide1 (GLP-1), a hypo-glycemic hormone enhancing glucose-dependent insulin secretion, and PYY in patients six weeks after RYGB when subjects were still obese, suggesting that this increased secretion was not dependent on the weight loss. They hypothesized that this increased hormonal secretion is due to the early direct contact between unabsorbed nutrients and intestinal L-cells, responsible of the production of these two hormones[197]. This way, insulin action is enhanced by GLP-1 and PYY increased

secretion. Also animal studies provided some hypotheses about the RYGB anti-diabetic effect. Troy et al. found increased levels of gluconeogenesis enzymes in the intestine of mice undergone a murine-counterpart of the human RYGB. The increased intestinal gluconeogenesis seems to activate a pathway through the portal vein and the glucose transporter GLUT-2, which is able to decrease glucose hepatic production and therefore increase hepatic insulin sensitivity[198].

All the proposed hypotheses described above are not exclusive. It is possible that some of the mechanisms discussed so far and some undiscovered pathways participate to the general anti-diabetic effect of the RYGB. At this stage, new target systems and clinical data are required to better address future research to understand the underlying physiological mechanisms of this effect.

IIId. Roux-en-Y gastric bypass, endocannabinoids and androgens: previous studies

Since more knowledge is needed about the modifications induced by RYGB surgery on different physiological systems to explain its metabolic effects, in the last years some clinical and animal studies were carried on investigating the alterations that RYGB could have on ECs and steroids.

Only one study so far investigated the relationship between metabolic changes induced by RYGB and ECs levels. In this study, performed in 2008 by Guijarro et al., they investigated the role of the ECS in three groups of diet-induced obese rats: RYGB rats, sham-operated rats and sham-operated pair fed rats[199]. They measured ECs in skeletal muscle and liver 14 and 28 days after the operation, corresponding approximately to one year and three years in humans respectively. They found a non significant increase of AEA concentration in both liver and skeletal muscle 14 days after the RYGB operation, despite the greater weight loss achieved compared to the other two groups. At the day 28, they found a significant decrease of AEA in both the analyzed tissues, following a further weight loss. A significant decrease of 2AG was also found at the day 28 only in the liver. The authors hypothesized that the decreased ECs concentrations were a consequence of all the improved metabolic parameters and the ECS could be implicated in the assessment of the physiological changes occurred after the RYGB[199].

On the contrary, several studies were carried out about the relationship between androgens functionality and RYGB. As described in the former chapter, obesity is characterized by decreased levels of androgens in men, likely due to a deregulation of the HPG axis, and different authors described the recovery of normal androgens levels in patients undergoing RYGB[115-117]. Hammoud et al measured total testosterone circulating levels in 22 obese men before and two years after RYGB. The average total testosterone was doubled after two years (before: 3,15 ± 2,01 ng/ml; two years after: 6,26 ± 1,85 ng/ml), furthermore they

found increases of free testosterone and SHBG; all these parameters were coherent with improvements in the sexual quality of life[116]. In another study, testosterone and DHEA levels were measured in obese men before, 3 months after, 6 months after and 1 year after a RYGB intervention. Testosterone levels increased significantly at all the study's time points, while no significant changes of DHEA concentrations were observed[117].

Taking into account all the clinical data discussed so far, RYGB seems to ameliorate androgens functions by increasing their circulating levels and, consequently, improve the sexual functions in RYGB patients.

IV. Aim of the project

Data discussed so far indicate that the ECS is strongly involved in the regulation of food intake and energy balance. Despite a meticulous characterization of this system in animals, human ECS has been not so accurately studied. To extend our understanding about the role of this system in human physiology and, in particular, its influence on the regulation of energy balance and the pathophysiology of obesity, recent studies are now focusing on the quantification of ECs in plasma.

So far, three main evidences have been found regarding the relationship between circulating ECs and obesity. First, plasma ECs levels are higher in obese patients compared to normo-weight patients, indicating an up-regulation of this system related to obesity. Second, after a diet-induced body weight loss, these circulating levels decrease, confirming that this up-regulation is directly linked to the obesity condition. Third, plasma AEA presents a pre-prandial peak and a post-prandial decrease, behaving as an orexigenic molecule.

Thus, in the second part of the thesis project we investigated the changes induced by RYGB intervention on ECS in obese patients. For this purpose, we used a sensitive and specific LC-MS/MS method previously validated following FDA guidelines for bioanalytical method validation[45] to quantify plasma levels of two endocannabinoids, AEA and 2AG, and two endocannabinoids related compounds, PEA and OEA, in 12 obese patients (BMI > 40 kg/m2 or BMI > 35 kg/m2 with obesity associated complications) undergoing RYGB. First, we measured fasting ECs in these patients before the surgery, one month after the surgery and six months after the surgery to assess variations of these levels in relation to different percentages of body weight loss obtained after the surgery. Second, performing blood withdrawals all over the day before and after a standardized meal at the three stages of the study, we assessed whether the ECs kinetics related to the food intake is subject to alterations.

Potential variations in ECs levels after RYGB, and therefore after a weight loss, can give significant clues about ECs functions and their involvement in the physiopathology of human obesity. Furthermore, some of the data discussed so far strongly suggest that ECs, particularly AEA, produced in the gastrointestinal tract could play an important role in the regulation of food intake and energy homeostasis in humans. Since this surgery is generating important and specific changes in the anatomy of the gastro-intestinal tract, the ECs levels variations related to food intake could be in part elucidated. In addition, in this study we looked at the correlations between circulating ECs levels and anthropometric parameters relative to visceral obesity.

Furthermore, in the same group of patients, we have been also interested in androgen measurement because obesity causes hypogonadism in men and in particular an important decrease of these circulating molecules likely due to HPG deregulation, as discussed in the former chapter. The decrease of circulating

androgens, and especially of testosterone, can lead impairment of the sexual function and obesity is one of the principal chronic pathological conditions characterized by this dysfunction. It is still not clear the direct link between obesity development and testosterone decrease, especially because the obesity-related deregulation of the HPG is sex-specific causing, on the contrary, increased circulating levels of testosterone in women. Furthermore, some clinical studies were carried out so far to investigate the recovery of androgens circulating normal levels and therefore an improved sexual function after a weight loss. Almost all the studies reported a significant increase of total testosterone after a weight loss obtained by both diet and bariatric surgery.

In order to better understand the beneficial effects of the RYGB and consequently of a weight loss on male sex steroids, we measured also fasting androgens, in particular testosterone and DHEA, in male patients. As well as ECs, androgens were evaluated at three different time point of the study: before, one month after and six months after the RYGB in relation to different percentage of weight loss, using a GC/MS method previously developed and validated.

Data resulting from testosterone and DHEA quantification will help to better characterized the androgen functions recovery after a weight loss induced by a specific surgery and therefore the recuperation of HPG functionality. Despite the existence of some data about this recovery, the underlying mechanisms are unclear. Resulting data from both ECs and androgens quantification at the three different time points of the study could be finally correlated, to assess if a direct link exists between these two kinds of circulating molecules. Indeed, animal and molecular studies showed that the ECS and the steroid system are interconnected, since important components of the ECS localize at all the three levels of the HPG and both systems have a key role in reproduction and sexual arousal. Moreover, it has been demonstrated in animals and humans a decrease of circulating testosterone under chronic THC treatments, results that were also confirmed in animals by chronically administering AEA.

With our study it will be possible to find out whether a correlation exists between circulating androgens and ECs, and if this correlation can be altered by body weight loss or other obesity-related parameters.

V. Materials and Methods

Va. Chemicals

Pure standards of endocannabinoids AEA, 2AG, PEA and OEA and ISTD d4-AEA, d5-2AG, d4-PEA and d2-OEA were from Cayman Chemical, Ann Arbor, MI. Pure standard and ISTD of testosterone and DHEA were from Sigma-Aldrich, St. Louis, MO.

Vb. Patients

i. Recruitment

So far, 12 obese patients (4 males and 8 females) out of 20 have completed the study. Subjects were recruited, after previous informed consent, at the CHU Haut Leveque in Pessac. The inclusion criteria were the following:

- Age between 18 and 60 years;

- Severe obesity with a BMI > 40 kg/m^2 or BMI > 35 kg/m^2 with associated obesity complications;

- Commitment for a prolonged post-operative follow-up;

- Effective contraception for pre-menopausal women.

The exclusion criteria were the following:

- Psychosis, severe depression or anxiety;

- Drugs addiction, tobacco addiction or chronic alcoholism;

- Associated pathologies involving a short-term prognosis;

- Patients without an adequate family or social support;

- Short-term pregnancy purpose;

- Presence of THC in urine.

For the inclusion, the metabolic phenotype was evaluated measuring weight and height for BMI assessment, waist circumference, concentration of fasting insulin, HbA1C, lipid profile (HDL cholesterol, triglycerides and LDL cholesterol) and liver enzymes. Visceral adiposity was measured with computed tomography (CT scan). Pre-operative patient characteristics are summarized in table 3.1.

Sex	8 Females/4 Males
Age (years)	39,92 ± 3,20
Body weight (kg)	136,8 ± 7,09
BMI (kg/m^2)	47,67 ± 1,65

Table 3.1: Pre-operative patients characteristics.

Behavioral phenotype was evaluated for each patients before the surgery, one month after the surgery and six months after the surgery using the French version of the *Beck depression questionnaire* to measure and exclude severity of depression, the *Three Factor Eating Questionnaire* to assess the eating behavior, the *Orwell 1997* questionnaire for the quality of life and the *Temperament and Character Inventory* for the personality evaluation.

ii. Hospitalization

Based on inclusion and exclusion criteria, 12 patients have been so far selected. These patients have been hospitalized, pre-operative parameters were assessed and the surgery was scheduled. 48 hours before the surgery, the blood withdrawals for the first time point of the study were carried on. Patients were hospitalized again one week, three weeks, one month and six months after the surgery to receive routine post-operative controls. One month and six months after the surgery were carried out other blood withdrawal for the second and third time point of the study.

Vc. Blood sampling

Blood withdrawals for ECs measurement were performed during different hours of the day at three different stages of the study: before the RYGB, one month after the surgery and six months after the surgery (figure 3.10). The first blood withdrawal (h: 9:00) was used to assess fasting circulating ECs and androgens concentrations. Following blood withdrawals were performed at 10:00, 11:00, and 11:30. At 12:00 patients were exposed to the food. At 12:20 the meal started and finished at 12:40. Other blood withdrawals were performed at the end of the first portion, at the end of the meal and 60, 120 and 180 minutes after the meal. The food was standardized for all the patients: each portion was 150gr of pasta served with 5gr of butter and 10 gr of grated cheese (265 kcal/portion). Blood was collected in heparin tubes and directly centrifuged at 2000 x g for 10 minutes at 4°C immediately after the withdrawal. Plasma was stored at -80° until the analysis.

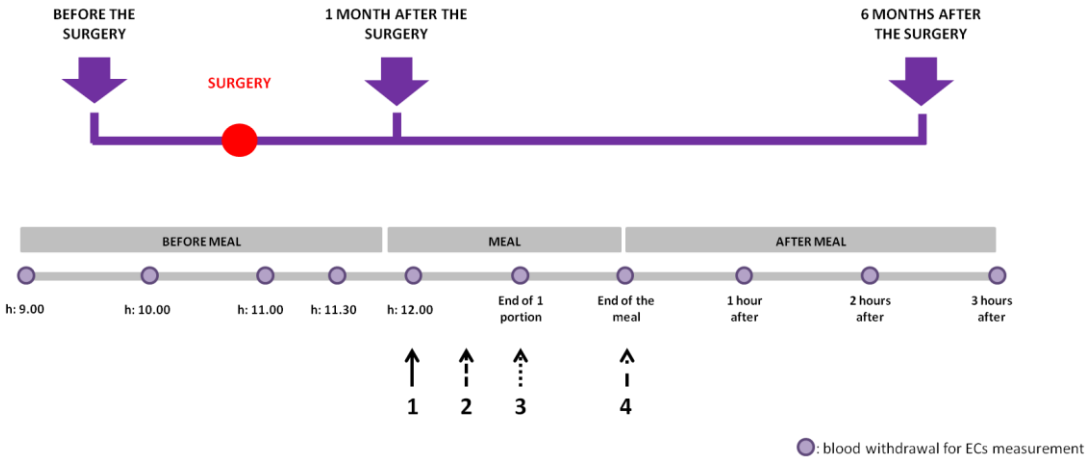


Figure 3.10: Design of the study. In three different stages (before the surgery, one month after the surgery and six months after the surgery) ten blood withdrawal were performed all over the day. Arrows indicate the phases of the meal: exposure to the food (**1**), meal start (**2**), end of the first portion (**3**) and end of the meal (**4**).

Vd. Endocannabinoids measurements

ECs measurement still represents an analytical challenge. These molecules are present at very low concentrations, especially in plasma, and their lipid nature makes the extraction and quantification even more problematic. Early quantification methods were developed between the end of 90s and the beginning of 2000s using GC/MS mainly to quantify these compounds in mice brain regions[200-201], but these methods were often laborious, since lipid molecules require derivatization to be analyzed with GC, and time consuming. In the last years, giving the increasing importance of the ECS in different clinical and research fields and particularly the emerging role of circulating ECs in human obesity, several more sensitive, fast and specific quantification methods were developed using LC-MS/MS[202-203]. This technique offers the best versatility, reliability, sensitivity and specificity required to measure these lipid analytes at very low concentrations. However, a dramatic discrepancy currently still exists between plasma ECs values found from different authors and research laboratories. It was shown that several different conditions could influence ECs concentrations and probably they represent the reasons behind the inconsistency of the values described so far[203-204]. Therefore, a precise standardization of blood withdrawal parameters, samples storage, laboratory materials, analytes extraction and LC-MS/MS analysis conditions, as well as a highly experienced staff, is required to avoid pitfalls that can affect ECs concentrations and to preserve the accuracy of the analytic method.

The LC-MS/MS method used for the ECs quantification in this thesis was previously developed at the Analytical Chemistry Platform of the Neurocentre Magendie INSERM U862 in Bordeaux. This method was validated following the FDA guidelines for bioanalytical method validation[45] for four analytes: AEA, 2AG, PEA and OEA, providing an adequate sensitivity and specificity to obtain accurate and precise ECs quantification in human plasma[22].

Endocannabinoids extraction. Lipids extraction was performed with a liquid-liquid method (LLE) modified from the Folch method. 1 ml of plasma was homogenized with chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2:1:1, v/v) containing ISTD. After liquid-liquid extraction, the organic phase was transferred in screw-cap Pyrex tubes and dried under a gentle stream of nitrogen. The dried lipid extracts were then pre-purified by open bed chromatography on silica columns. After the sample load, a first washing step was performed with chloroform/methanol 99:1 while the eluting step was with chloroform/methanol 9:1. The eluted fractions transferred in 1,5 ml eppendorf were finally dried under nitrogen, reconstituted in 65µl of methanol and transferred in autosampler vials for the LC-MS/MS analysis[22].

Liquid chromatography. Chromatographic analysis was performed with a Surveyor LC Pump Plus apparatus (Thermo-Scientific, Waltham, MA, USA). Analytes separation was achieved using an analytic column Supelco Discovery C18 15 cm x 4,6 mm x 5µm, a reverse phase analytic column with a non-polar stationary

phase suitable for lipid analytes such as ECs, equipped with a column-guard Phenomenex Analytical Guard Cartridge C18 4 x 3 mm; both analytic column and guard-column were kept in the column oven set at 40°C. The chromatographic separation for AEA, 2AG, PEA and OEA was achieved using 1ml/min polar mobile phases (A: H_2O 100% and B: methanol with 0,1% of acetic acid) usually adequate for reverse phase chromatography, and an isocratic gradient (15% A, 85% B), suitable for compounds with similar polarity, with excellent resolution. The overall chromatographic run was 25 minutes.

Mass spectrometry. Mass spectrometry analysis was performed with a TSQ Quantum Access triple quadrupole instrument (Thermo-Scientific, Waltham, MA, USA). Eluted analytes carried from the analytical column outflow were ionized using an APCI ionization source, the most suitable to ionize analytes in this range of molecular weight and to use a mobile phase flow of 1 ml/min, operating in positive ion mode at a temperature of 350°C. The ionization nebulizer gas was nitrogen, while the collision gas for analytes fragmentation during the MS/MS analysis was pure argon. All the precursor ions, product ions and collision energy previously optimized for each analyte are summarized in table 3.2. Mass spectrometer operated in SRM mode.

Quantification. Stock solutions and working solution were prepared for each analyte in methanol and stored at -20°C. A linear six-points calibration curve was prepared from the working solutions ranged from 0.15 pmol to 7,5 pmol for AEA and from 1,5 pmol to 75 pmol for 2AG, PEA and OEA. The isotopic dilution quantification method by linear regression, described in the former chapter, was the chosen quantification mode.

Compound	Molecular weight	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
AEA	347,5	348,2	287,05	13
d4-AEA	351,6	352,2	287,12	14
2AG	378,6	379,2	287,01	14
d5-2AG	383,6	384,2	287	16
PEA	299,5	300,2	62,35	16
d4-PEA	303,5	304,2	62,32	16
OEA	325,5	326,2	62,2	18
d2-OEA	327,5	328,2	62,29	18

Table 3.2: Molecular parameters used for the LC-MS/MS analysis.

Ve. Androgens measurements

Androgen quantification was performed with a GC/MS method previously developed and validated[205-206] and routinely used at the Analytical Chemistry Platform of the Neurocentre Magendie INSERM U862 in Bordeaux. Despite LC-MS/MS is becoming more used in clinical and research laboratories for steroids quantification, GC/MS represents still the *gold standard* technique for these hormones in the clinical routine.

Steroids extraction. Extraction of steroid was performed with a SPE followed by derivatization. ISTD were added to 300µl of plasma then mixed with 500 µl of methanol/H_2O (75/25, v/v) and 500 µl of pure methanol. After centrifugation (10000 rpm, 10 min, 4°C) supernatants were transferred in glass tubes, partially dried under a stream of nitrogen and diluted in 2,5 ml of methanol/H2O (5/95, v/v). Steroids were extracted with SPE using reverse-phase Bond Elut C18 100mg 3 ml cartridges (Agilent, France) with following steps:

- activation of C18 cartridges with 2 ml of methanol;

- washing with 2 ml of methanol/H_2O (5/95, v/v);

- samples loading;

- washing with 2 ml of methanol/H_2O (5/95, v/v);

- second washing with 2 ml of methanol/H_2O (50/50, v/v)

The steroid fraction was finally eluted with methanol (2 ml) into screw-cap pyrex tubes and evaporated to dryness at 50°C under a nitrogen stream to prepare for derivatization[205-206].

Derivatization. The derivatization phase, as explained in the former chapter, is required in GC/MS analysis to make compounds more volatile. Plasma dried extracts were derivatized by a two-step procedure. First, pentafluorobenzyloxime was added to favor the negative chemical ionization detection. After 1 hour incubation at 70°C, trimethylsilyl was added to each sample to reach sensitivity, selectivity and reproducibility for the simultaneous detection of the analytes. Samples were incubated at 70°C for 20 minutes, transferred in autosampler vials and then injected in the GC/MS system[205-206] (figure 3.11).

GC/MS analysis. 1 µl of derivatized samples were injected into a GCMS-QP2010 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) through an autosampler. Analytes separation was achieved using a 15 m Rtx-5Sil MS W/Integra Guard capillary column (Restek, France) with a 0.25 mm inside diameter and 0.1 µm film thickness. The carried gas was ultra-high pure helium (Linde Gas, France) at linear velocity (60 cm/s). The employed injector temperature was 260°C and splitless mode was used for 0,5 minutes after the

injection. Capillary column temperature was maintained at 160°C for 1 minute. One minute after the samples injection column oven was warmed at 230°C with a rate of 60°C/min; temperature was then raised to 260° at a rate of 4°C/min and then to 290°C at 60°/min. Transfer-line temperature was kept at 290°C. The total chromatographic run was 15 minutes.

Analytes ionization was performed with negative ion chemical ionization mode. Ion source temperature was 200°C and the ion source pressure was 0,7-0,8 Torr; methane (research grade 5.0, 99.9995%; Air Products, France) was used as reactant gas. Emission current was set at 60 µA and electron energy was 70 eV. Mass spectrometer operated in SIM mode.

Quantification. Stock solutions and working solution were prepared for each analyte in methanol and stored at -20°C. BSA 4% was used as matrix for a seven-point calibration curve ranging from 0,5 to 32 pg for each analytes. The pure BSA solution represented the "zero point". SPE procedure described above was performed for each calibration curve point and the zero point. The isotopic dilution quantification method by linear regression was the chosen quantification mode.

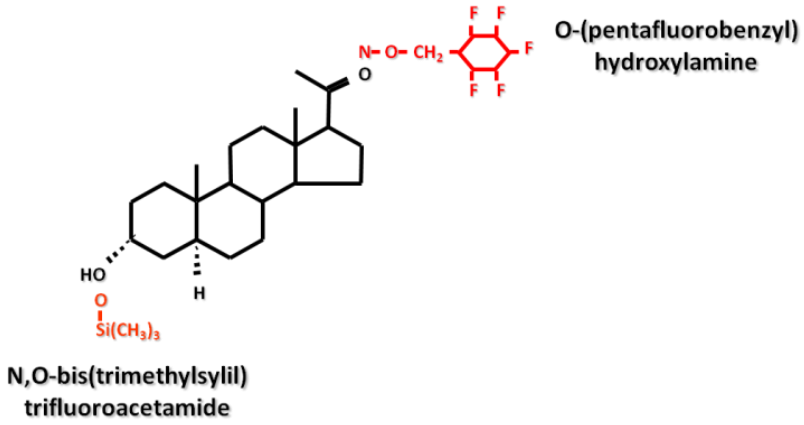


Figure 3.11: Steroids derivatization model.

Vf. Anthropometric and biochemical measurements

Patients weight was controlled with a balance having a precision of 0,1 kg and height with a stadiometer with an accuracy of 0,5 cm. Waist circumference was measured between the upper iliac spines and the last ribs with a measuring tape.

Vg. Statistics

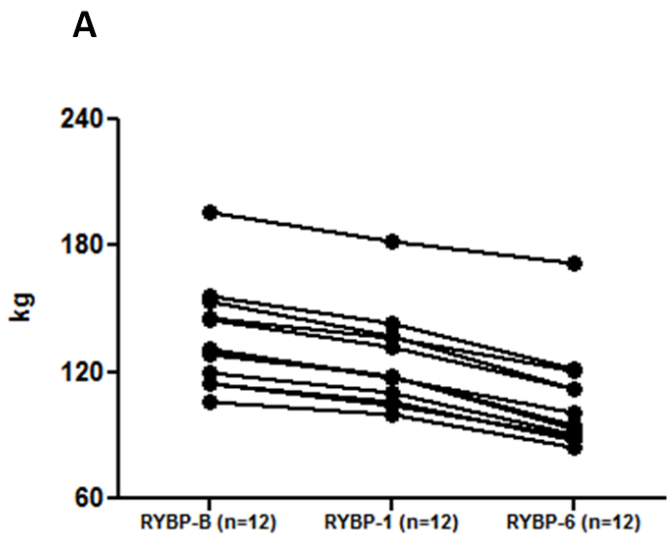
Data analysis was performed with GraphPad Prism 5 program. Results are expressed by mean ± SEM. Area under the curve (AUC) was calculated using the trapezoidal method to quantify changes of ECs levels in the kinetics. Data were analyzed with One Way ANOVA (fasting ECs, fasting androgens, AUC) or Two Way ANOVA (kinetics); performed post-test was Tukey. Mean differences were considered statistically significant when $p<0,05$ (*$p<0,05$; **$p<0.005$; ***$p<0.0005$).

VI. Preliminary results

The initial project was designed to include 20 patients. Considering surgery, hospitalization and follow-up duration, estimated project time was two years. At the present moment, the results presented in this thesis concerning ECs measurements are relative to 12 patients out of 20 who completed the study, while androgens measurements were carried out so far for 2 out of 4 male patients.

VIa. Patients weight loss achievement

Pre-surgery patients body weight was on average 136,79 ± 7,09 kg; one month after the surgery patients body weight on average was 125,3 ± 6,60 kg while six months after the surgery it was 106,7 ± 7,01 kg (figure 3.12).



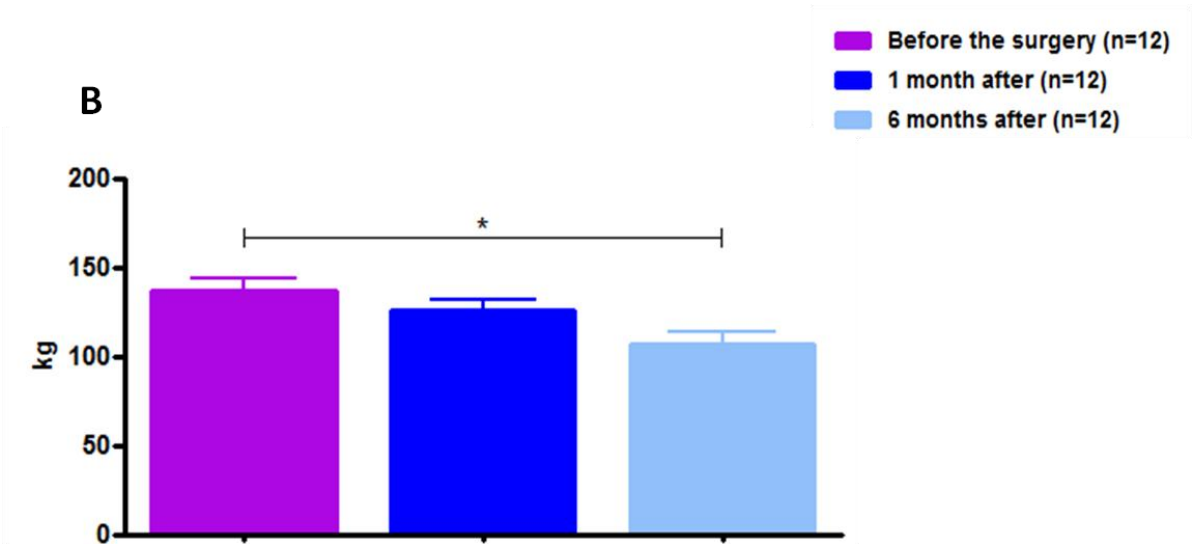


Figure 3.12: Body weight changes for each patient, in detail (**A**). Changes of patients weight on average (**B**).

Patients achieved a body weight loss of the 8,39 ± 0,42% of total body weight one month after the surgery and 22,38 ± 1,27% of total body weight six months after the surgery (figure 3.13). Different literature data concerning RYGB showed a body weight loss of 37% on average one year after the surgery[111]; therefore, since our patients lost more than 20% of the total body weight six months after the surgery, they are responding as expected to the treatment.

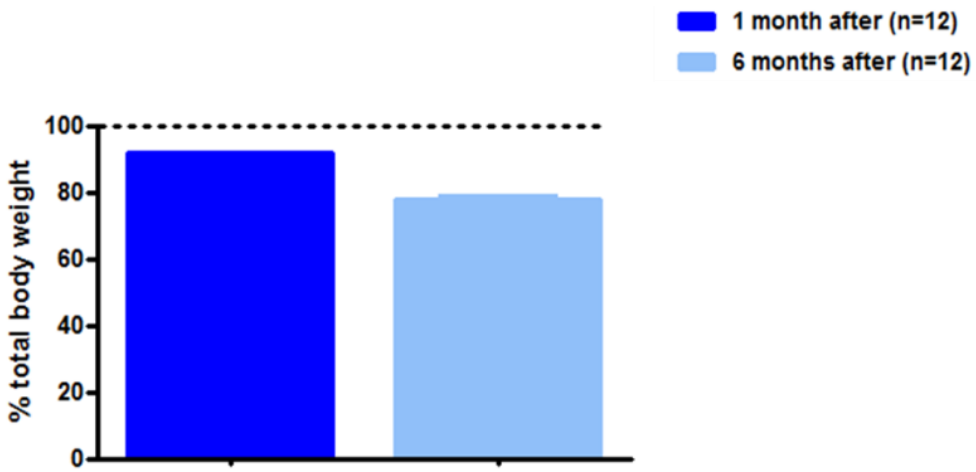


Figure 3.13: Patients body weight loss in percentage of total body weight.

As well as the body weight also the BMI was markedly reduced after the RYGB. Pre-operative BMI on average was 47,67 ± 1,65 kg/m^2. After the surgery BMI was significantly reduced: 43,90 ± 1,51 kg/m^2 one month after and 37,22 ± 1,56 kg/m^2 six months after (figure 3.14).

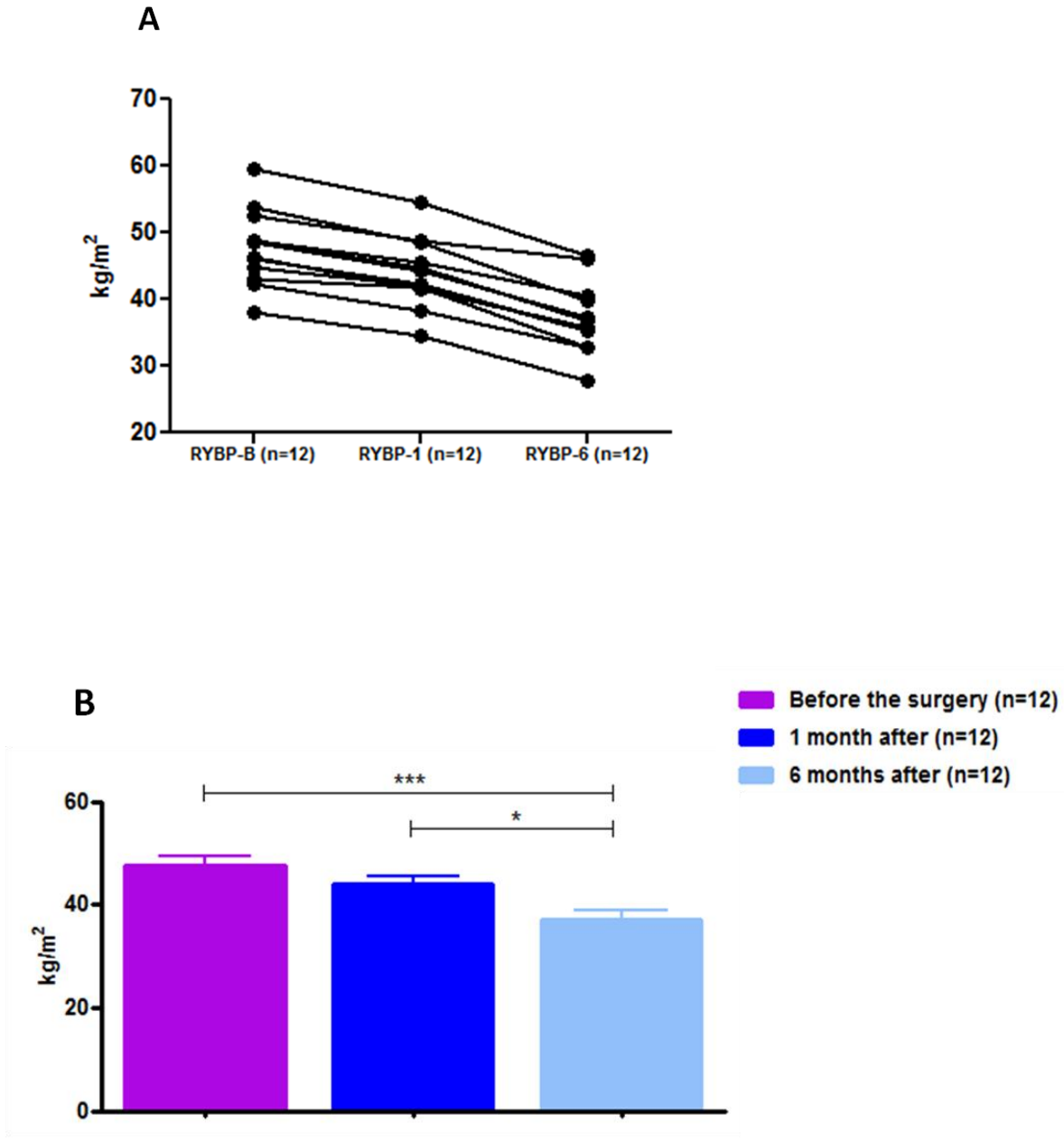


Figure 3.14: BMI changes for each patient, in detail (**A**). Changes of patients BMI on average (**B**).

VIb. Circulating endocannabinoids levels in obese RYGB patients

AEA. We observed that plasma AEA fasting levels showed a tendency to increase one month after the RYGB. Conversely, six months after, AEA circulating levels tend to decrease (figure 3.15).

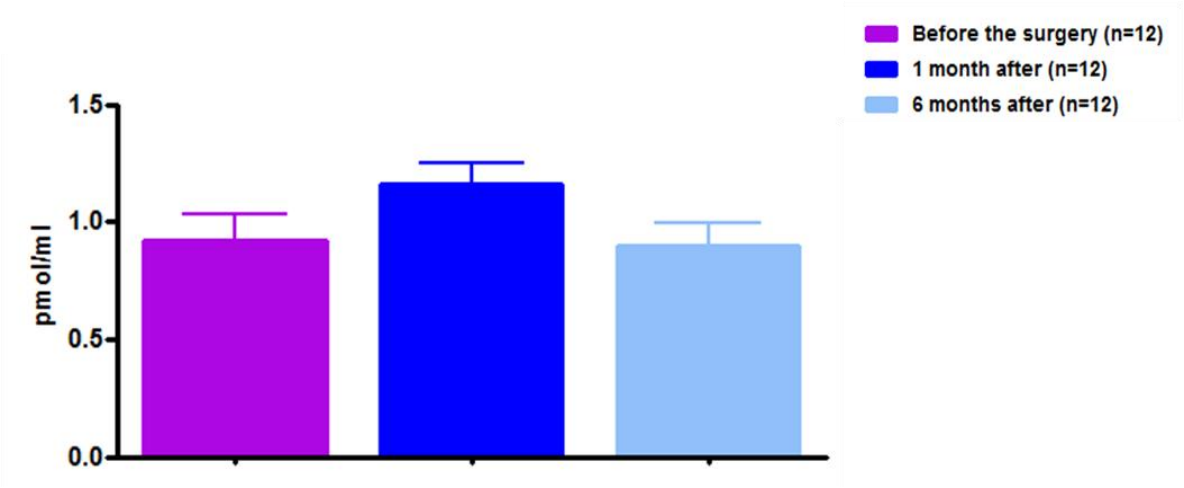
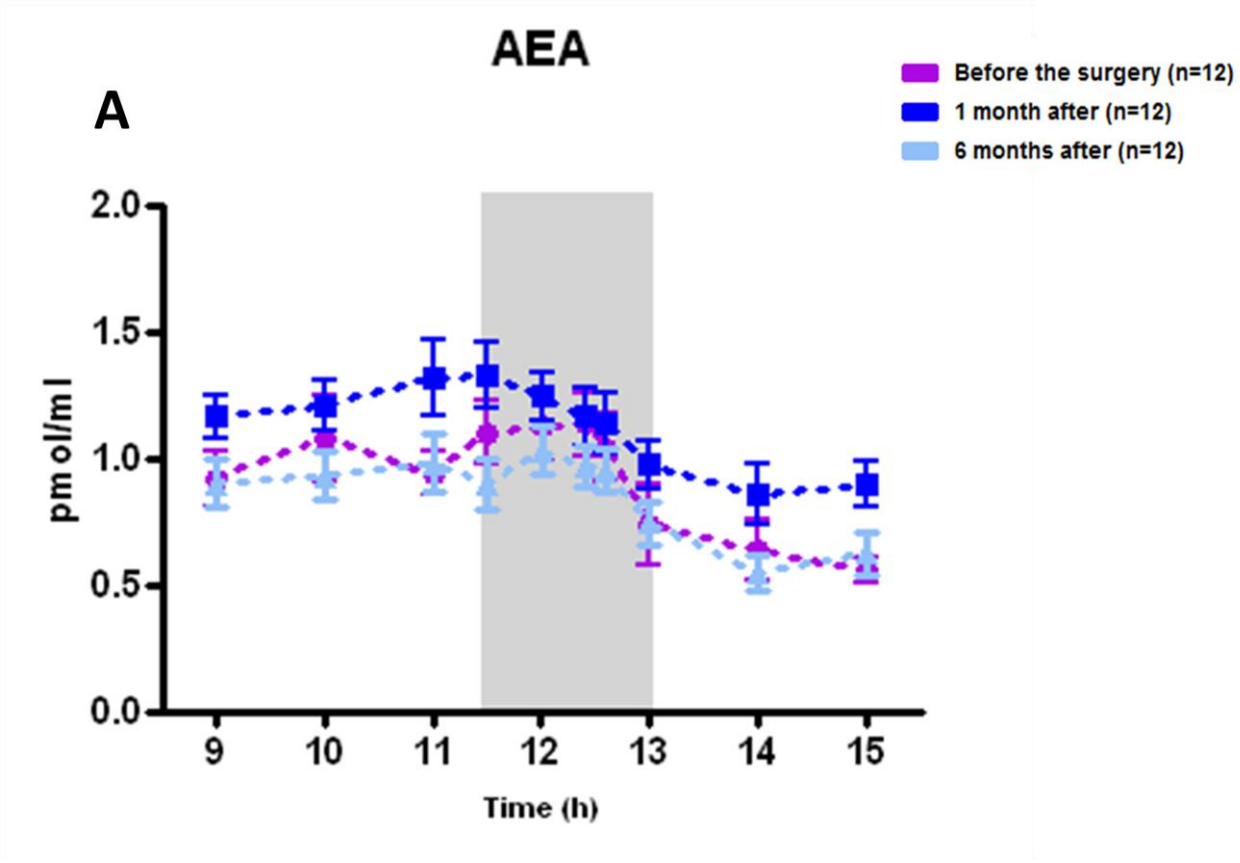


Figure 3.15: Overnight fasting AEA levels.

Analyzing AEA meal related kinetics we found the same tendency. One month after the RYGB subjects tended to have higher levels of circulating AEA. Differently, six months after the surgery AEA levels went back to pre-surgery values (figure 3.16 A-B), as demonstrated from the significant differences observed between the AUC (figure 3.16 C). At this time-point we observed also a tendency for AEA to increase right before the meal and decrease after the meal, showing kinetics similar to the normal weight subjects (figure 3.16 D). No correlations have been found between circulating AEA levels and BMI (figure 3.17).



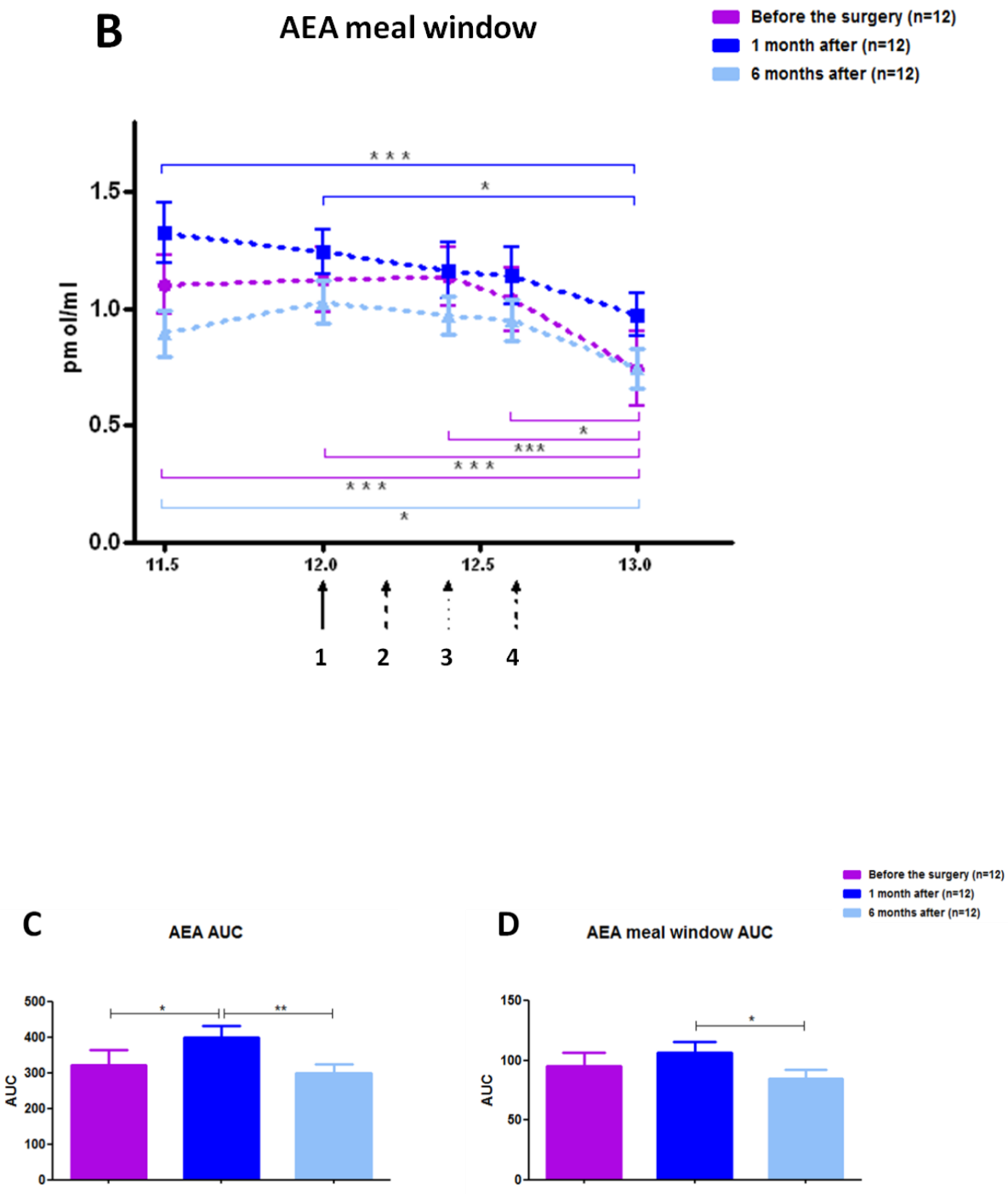
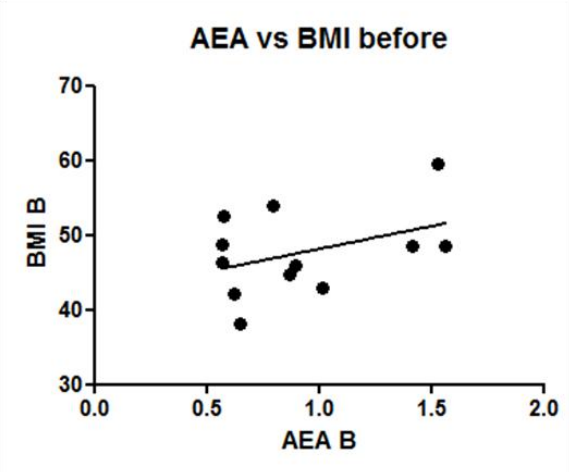
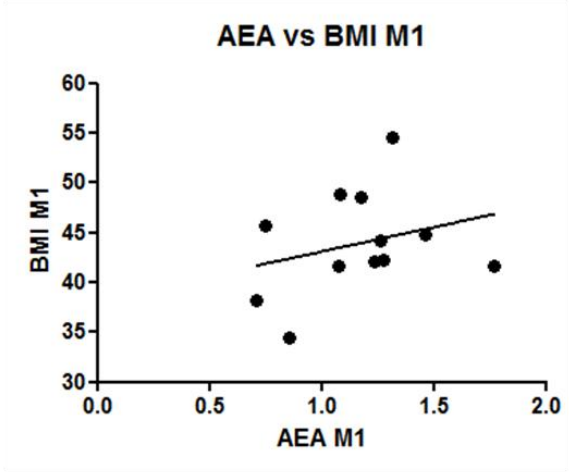


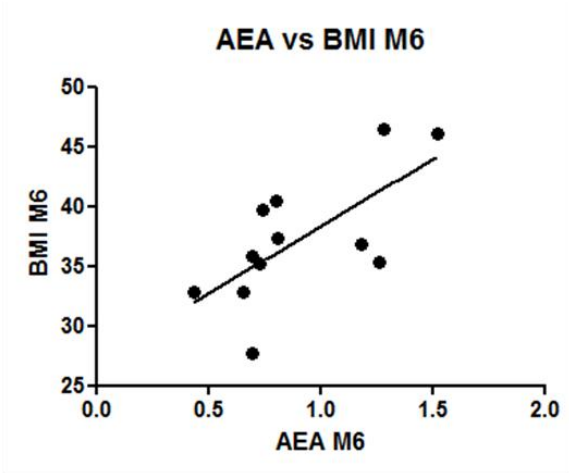
Figure 3.16: Whole daily dynamic AEA concentrations (**A**). In detail, AEA dynamic levels during the meal and relative AUC. Arrows indicate the phases of the meal 1: exposure to the food; 2: meal start; 3: end of the first portion; 4: end of the meal (**B**). Complete kinetics AUC (**C**). Meal window kinetics AUC (**D**).



$r^2 = 0,1626$



$r^2 = 0,07812$



$r^2 = 0,4638$

Figure 3.17: Correlation between AEA circulating levels and BMI.

2AG. 2AG circulating fasting levels are not affected one month after the surgery; however, we observed a tendency of these values to increase six months after the surgery (figure 3.18).

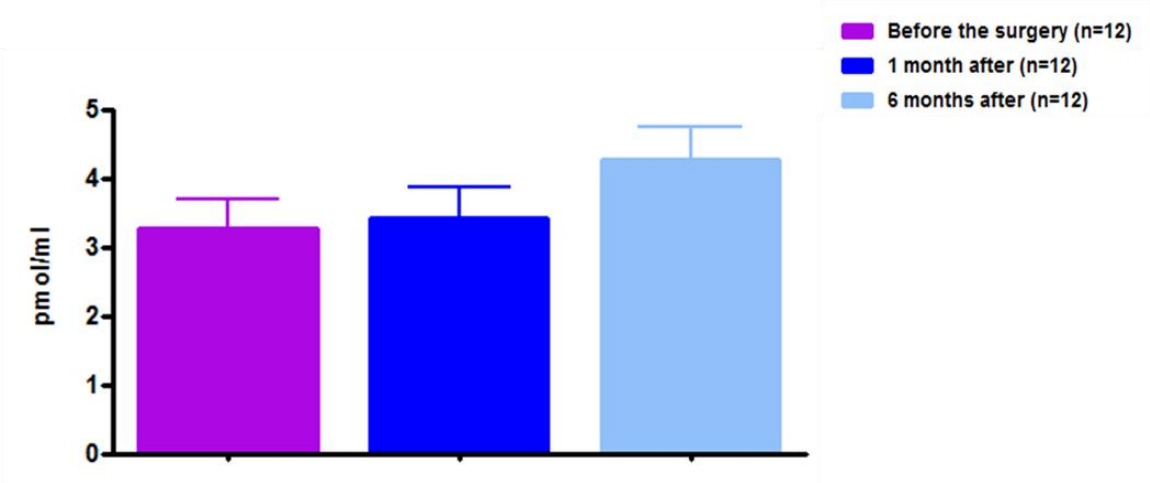
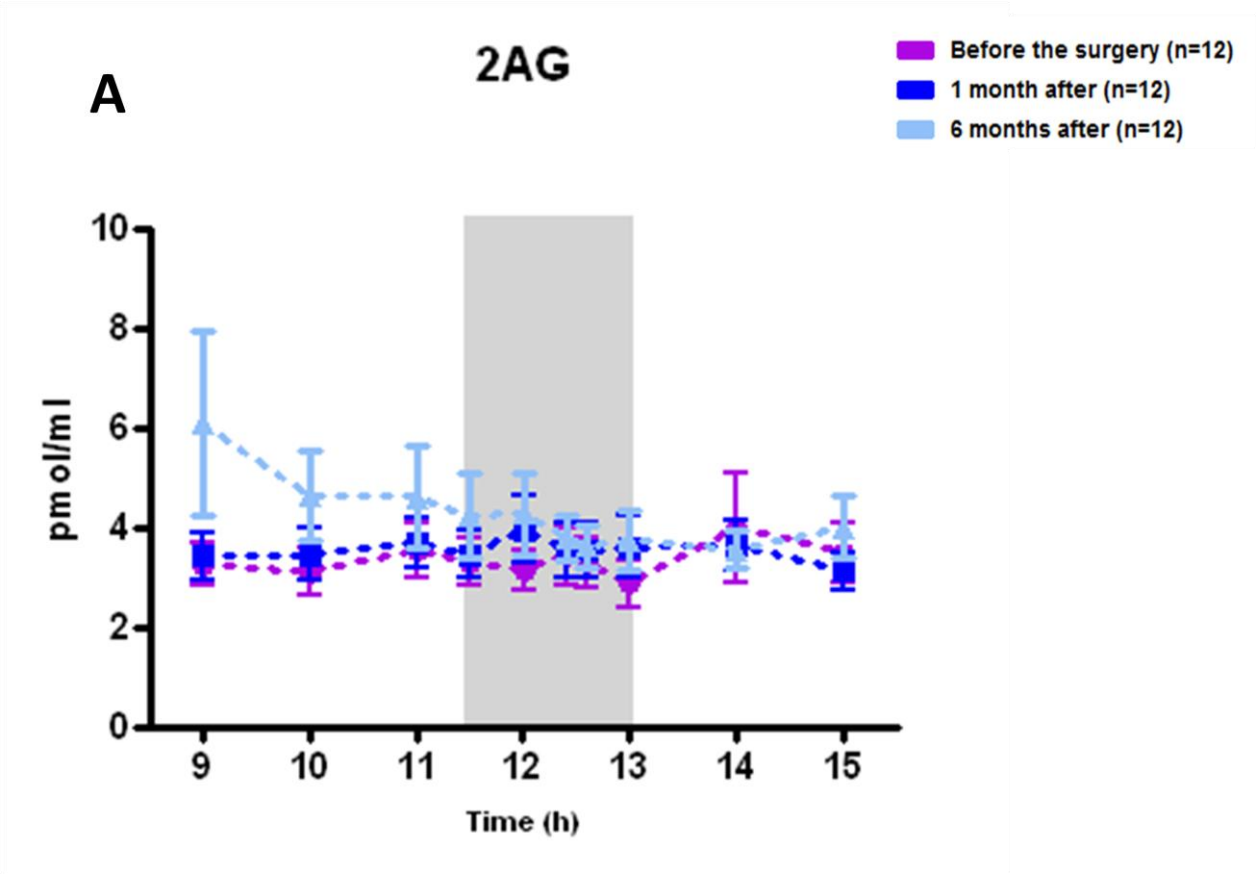


Figure 3.18: Overnight fasting 2AG levels.

We did not observe significant variations in the 2AG kinetics (figure 3.19 A-B-C-D), similarly to findings previously published[21]. Similarly, no correlation has been found between circulating 2AG and BMI (figure 3.20).



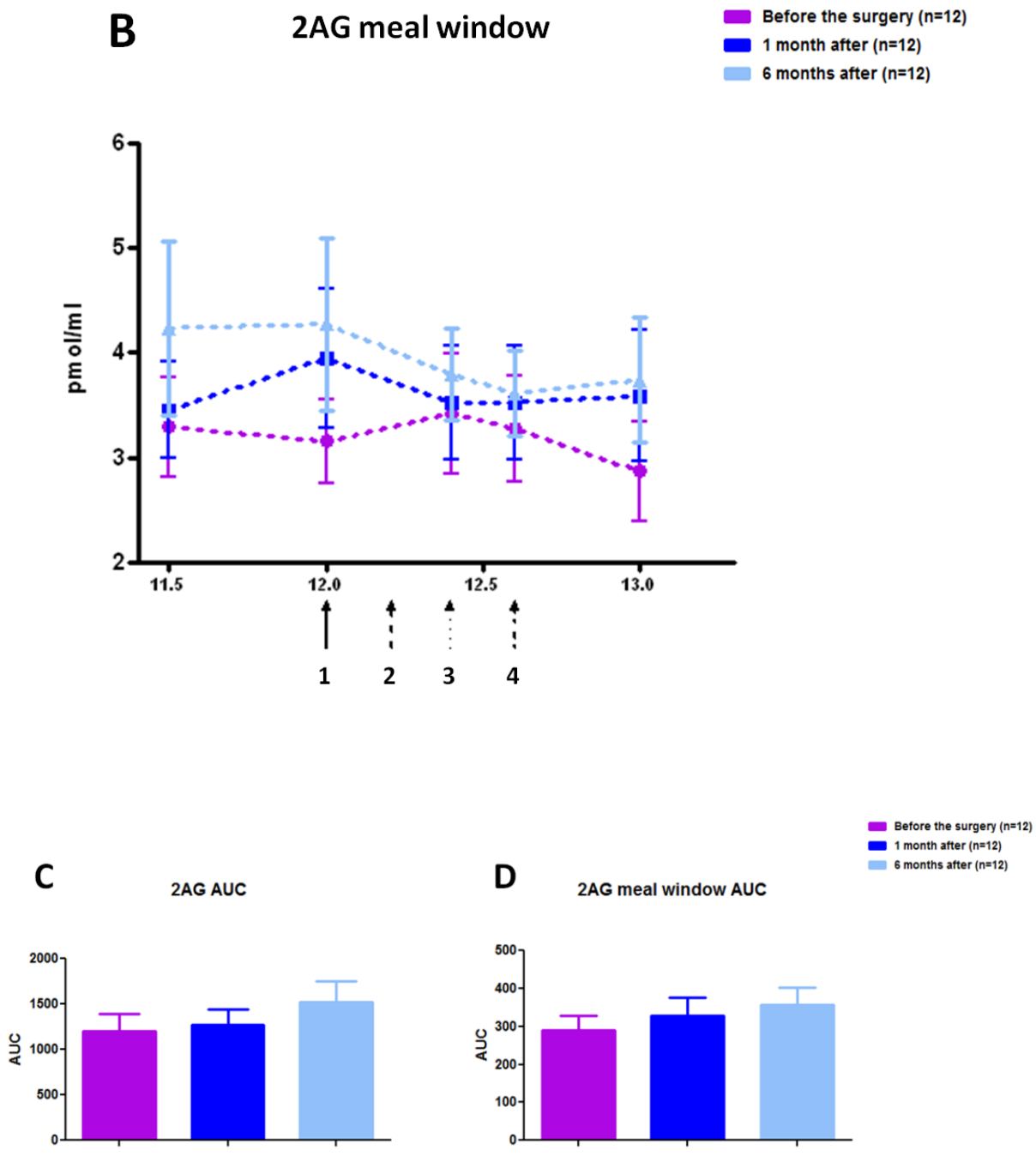


Figure 3.19: Whole daily dynamic 2AG concentrations (**A**). In detail, 2AG levels during the meal and relative AUC. Arrows indicate the phases of the meal 1: exposure to the food; 2: meal start; 3: end of the first portion; 4: end of the meal (**B**). Complete kinetics AUC (**C**). Meal window kinetics AUC (**D**).

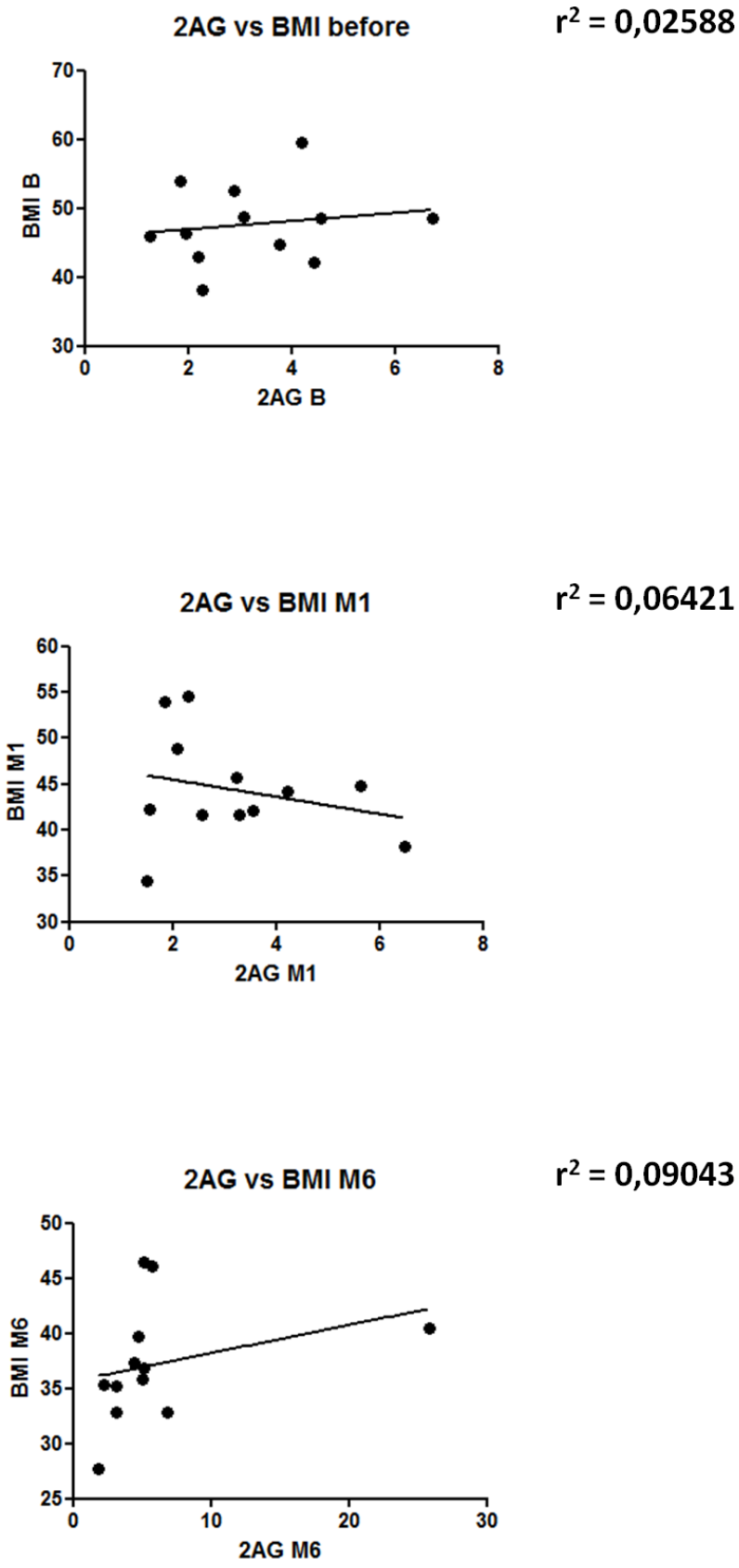


Figure 3.20: Correlation between 2AG circulating levels and BMI.

PEA. We did not observe significant differences one month after the surgery for fasting levels of PEA. Six months after the surgery there is a tendency to a decrease (figure 3.21).

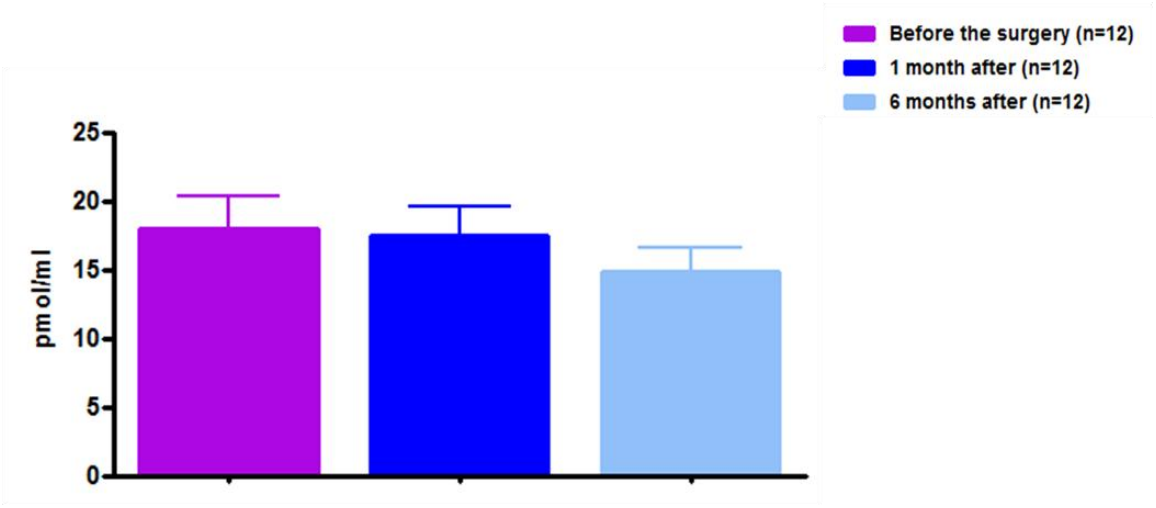
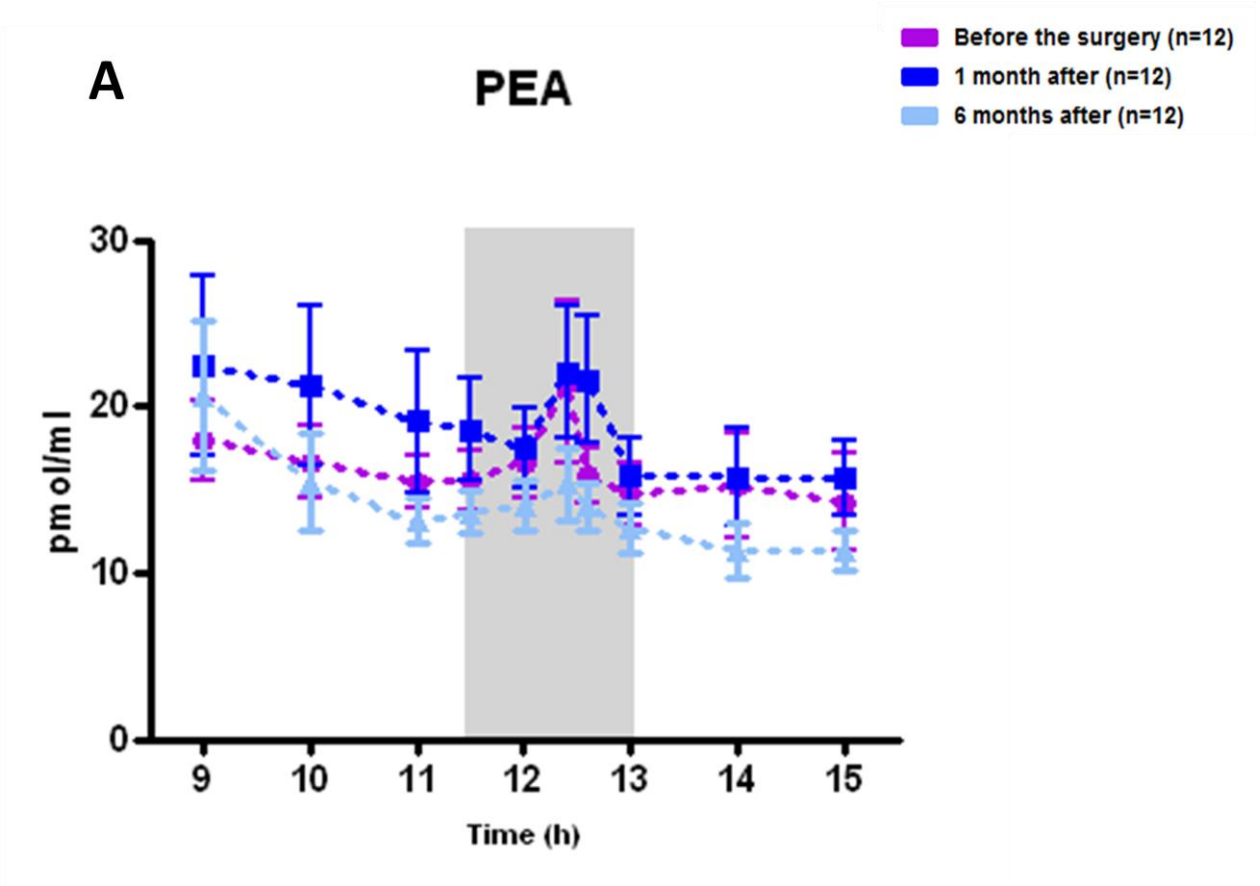


Figure 3.21: Overnight fasting PEA levels.

Circulating PEA levels are overall lower after 6 months compared to 1 month after the RYGB (figure 3.22 A-B), as we found significant differences in the AUC (figure 3.22 C-D). No significant variations related to the feeding status have been observed as well as no correlation between plasma PEA levels and patients BMI was found (figure 3.23).



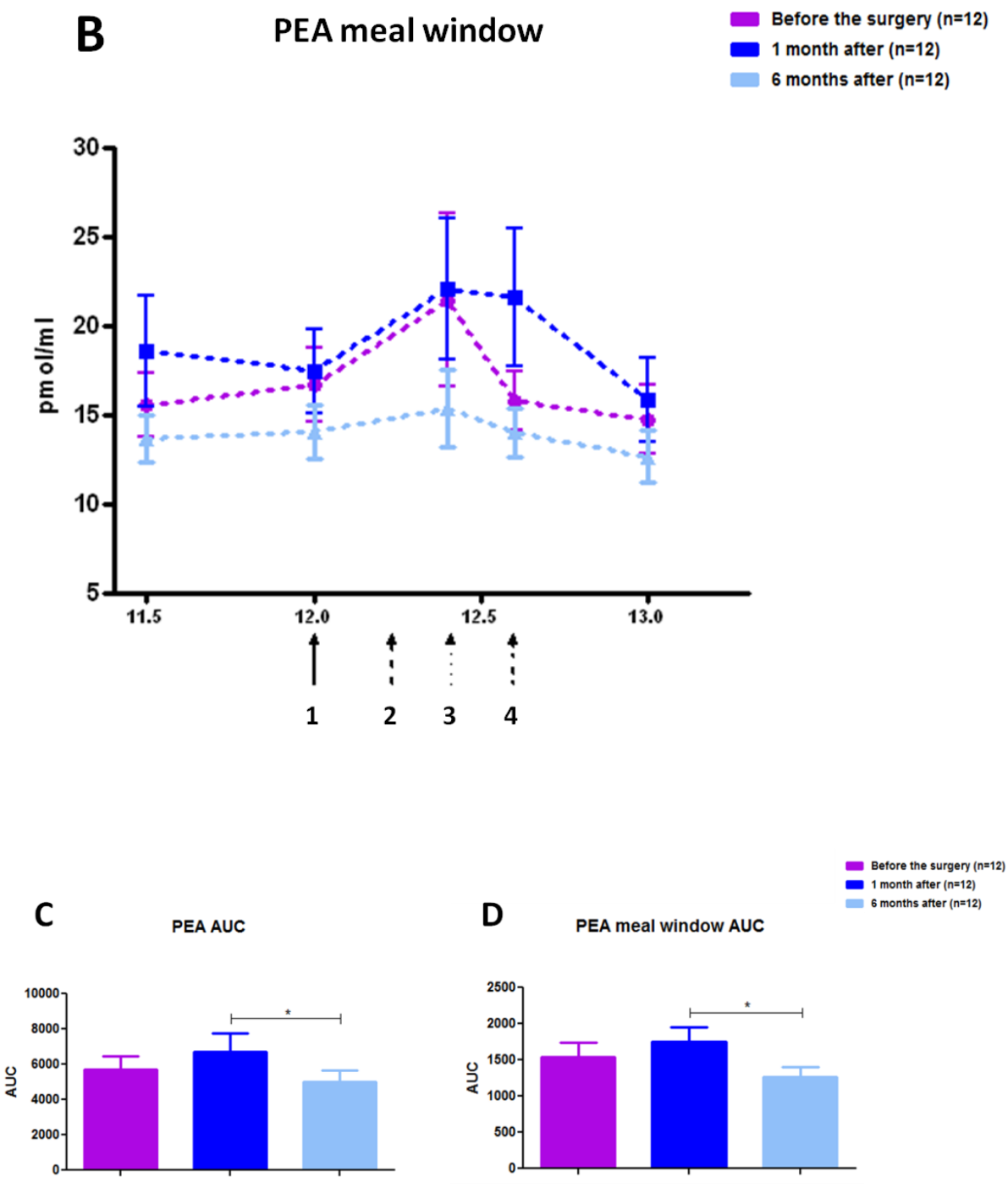
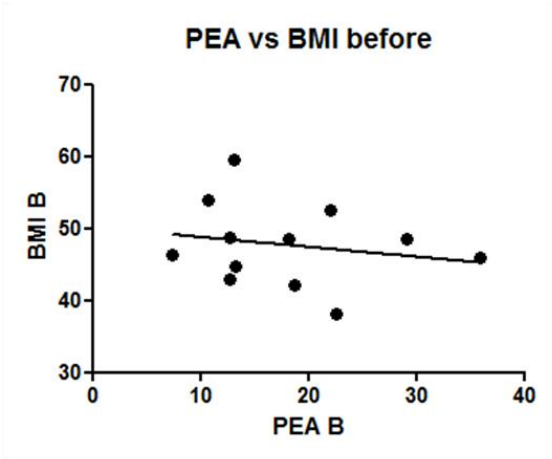


Figure 3.22: PEA concentrations across the day (**A**). In detail, PEA levels during the meal and relative AUC. Arrows indicate the phases of the meal 1: exposure to the food; 2: meal start; 3: end of the first portion; 4: end of the meal (**B**). Complete kinetics AUC (**C**). Meal window kinetics AUC (**D**).



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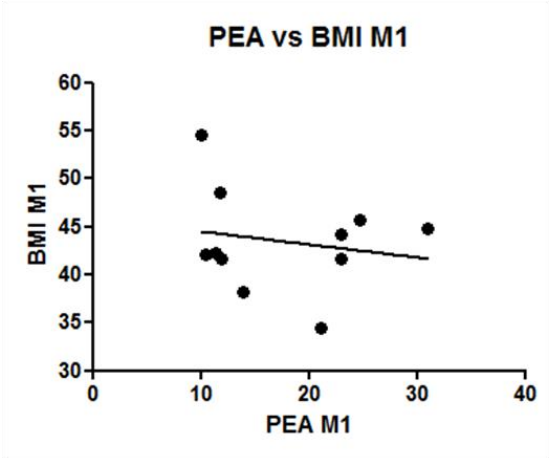
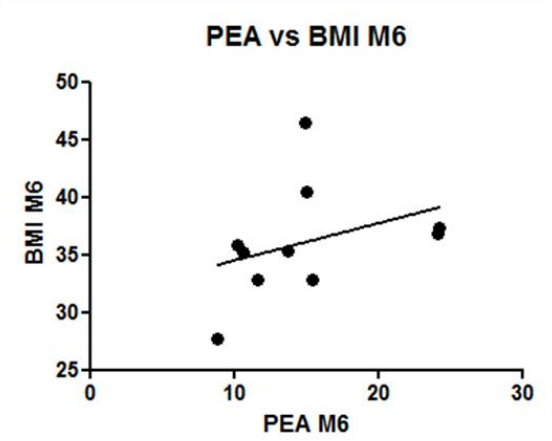


$r^2 = 0,03510$



$r^2 = 0,1233$

Figure 3.23: Correlation between PEA circulating levels and BMI.

OEA. Differently from AEA, fasting circulating OEA levels showed a tendency to increase both one and six months after the RYGB compared to pre-operative levels (figure 3.24).

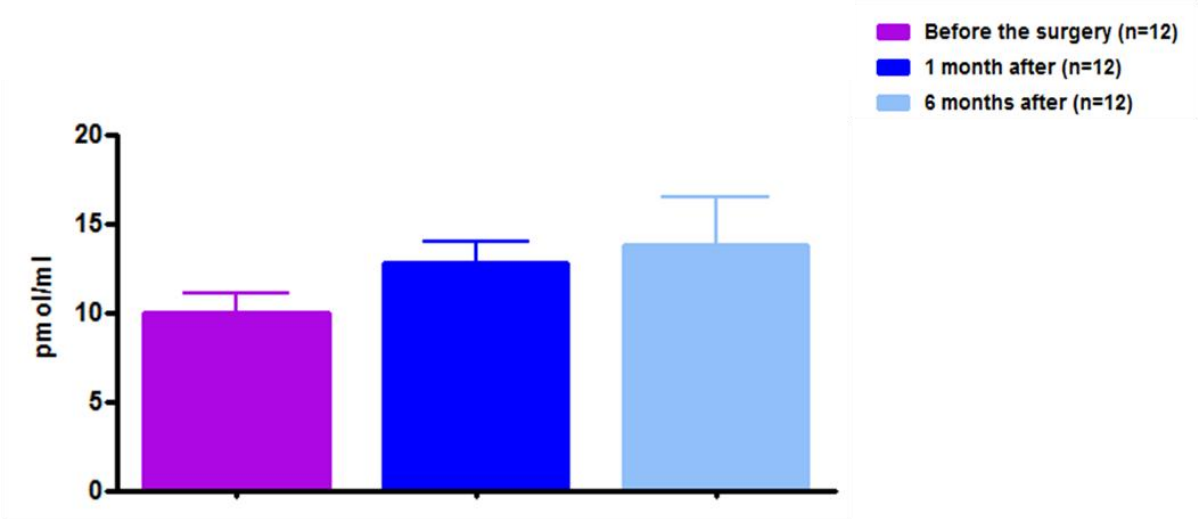
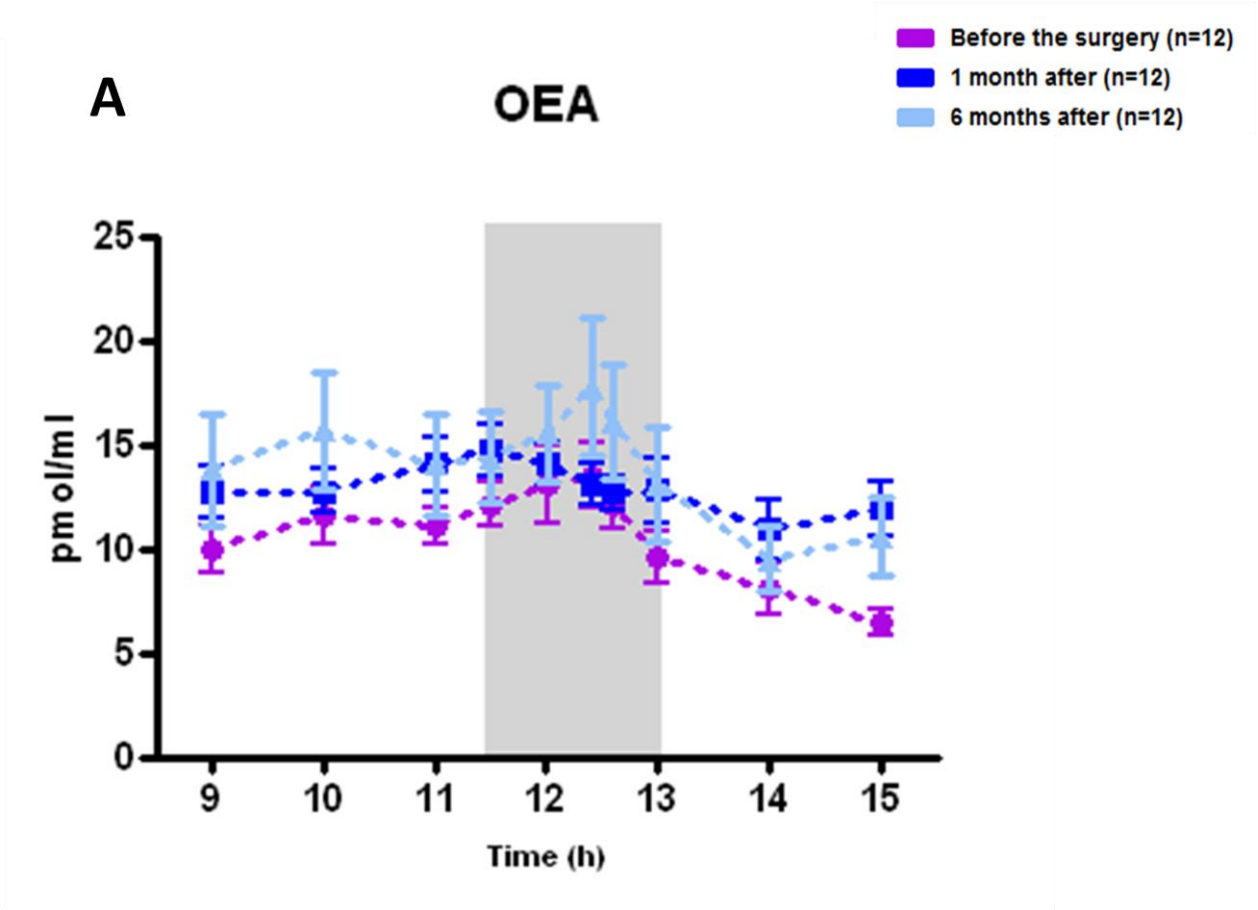


Figure 3.24: Overnight fasting OEA levels.

Concerning the kinetics, while we did not find significant differences between before and 1 month after the GYBP, at 6 months subjects tend to have increased OEA levels, which decrease after the meal (figure 3.25). No correlation has been found between OEA levels and BMI (figure 3.26).



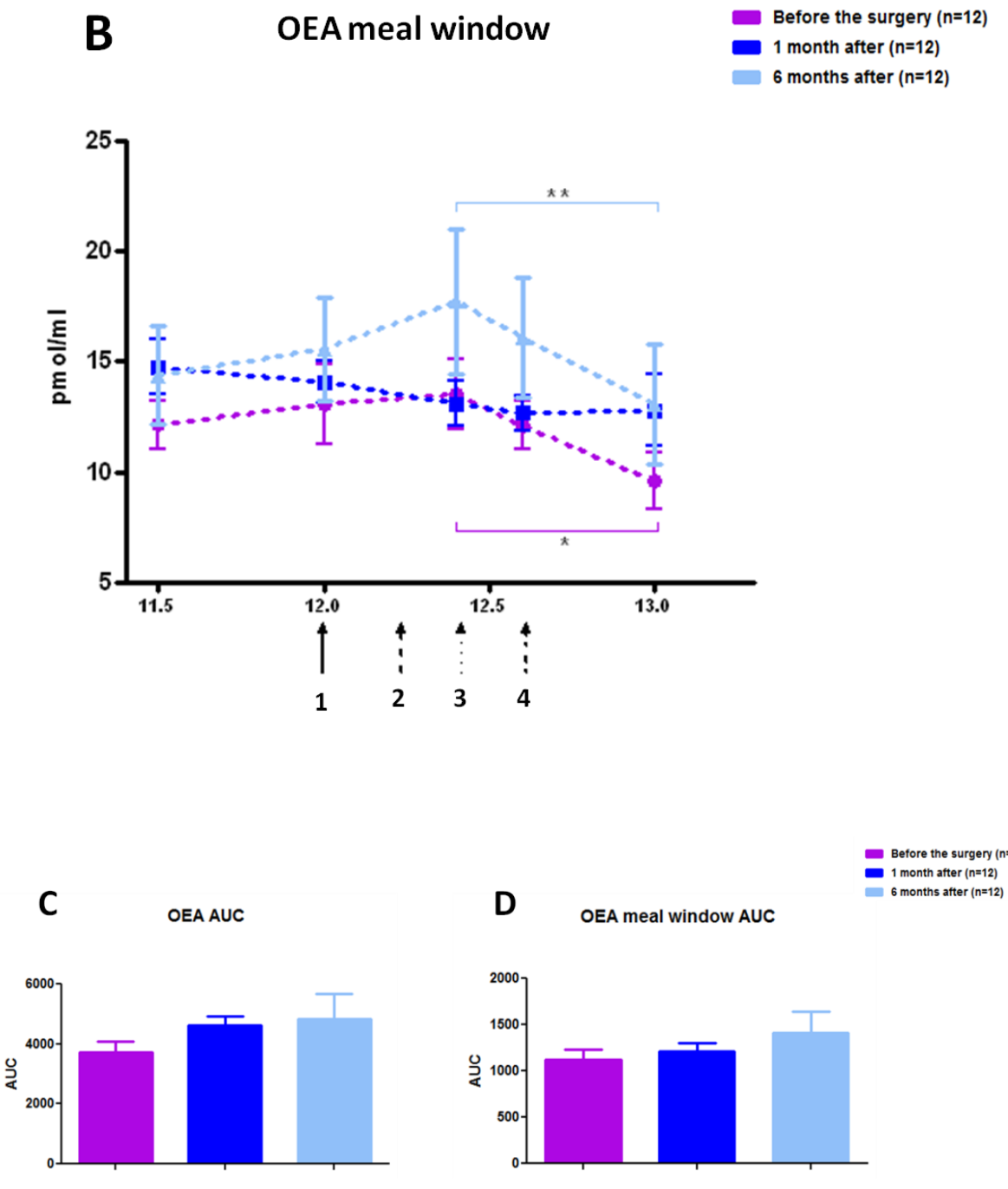


Figure 3.25: OEA concentrations across the day (**A**). In detail, OEA levels during the meal and relative AUC. Arrows indicate the phases of the meal 1: exposition to the food; 2: meal start; 3: end of the first portion; 4: end of the meal (**B**). Complete kinetics AUC (**C**). Meal window kinetics AUC (**D**).

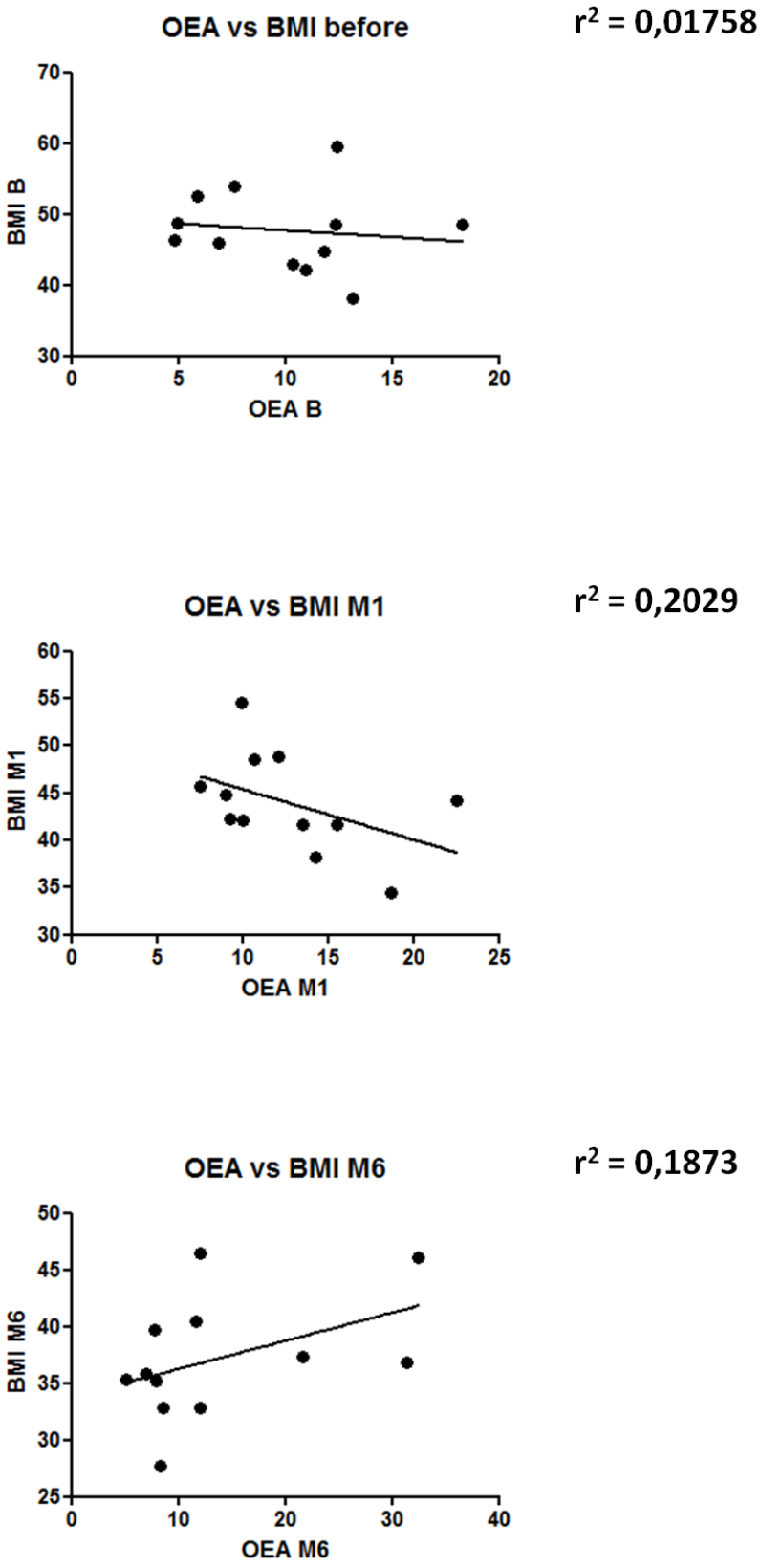


Figure 3.26: Correlation between OEA circulating levels and BMI.

VIc. Circulating androgens levels in obese RYGB patients

Testosterone. Testosterone overnight fasting circulating levels tend to increase one month after the surgery. Six months after the surgery we observed a further increase, at this time point testosterone plasma levels are significantly different from testosterone levels before the surgery (figure 3.27).

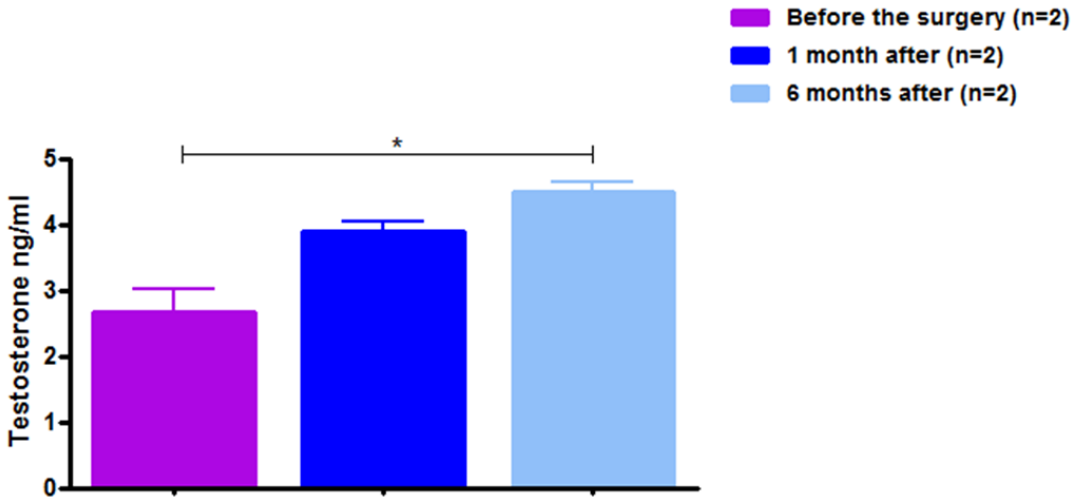


Figure 3.27: Overnight fasting testosterone levels.

DHEA. Similarly, DHEA overnight fasting concentrations tend to increase after the surgery (figure 3.28).

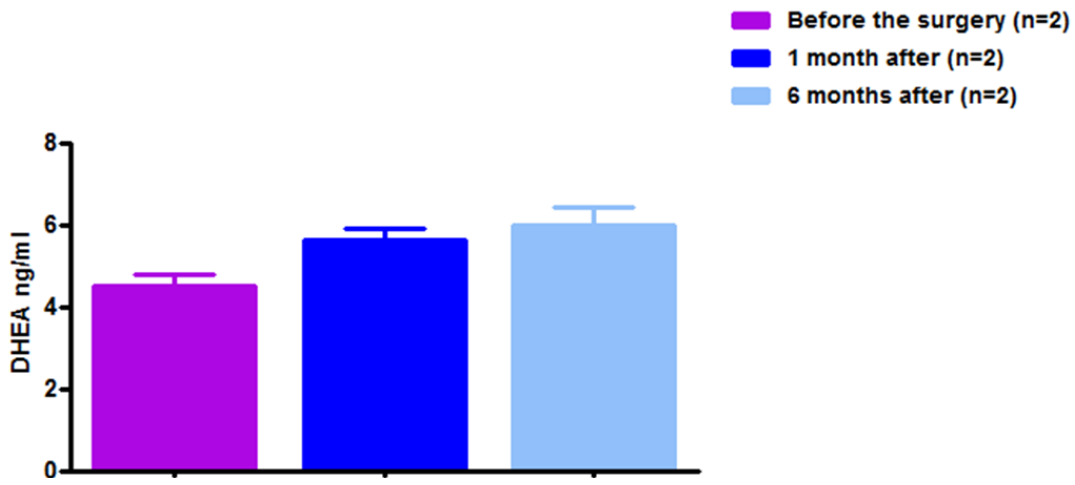


Figure 3.28: Overnight fasting DHEA levels.

VII. Discussion, conclusion and future perspective

Currently, obesity is an important health threat worldwide since it leads to fatal co-morbidities and a consequent reduction of life-span[4]. Apart from the behavioral therapy, including diet, exercise and psychological support, two therapeutic approaches are available for the management of obesity: drug therapy and bariatric surgery. However, only one drug is currently available for the pharmacological therapy of this condition[180]. Bariatric surgery is currently the most effective anti-obesity long-term treatment in reducing body weight and obesity related co-morbidities. The most performed type of bariatric surgery is the RYGB[23], since it ensures the best body weight loss results and the less amount of body weight regain. Furthermore, patients undergoing this surgery present a surprisingly high percentage of T2DM remission, approximately the 80% of subjects[111]. Despite this phenomenon has been extensively examined, the molecular and physiological mechanisms underlying this remission are still poorly understood[207]. To improve the knowledge about effects of anatomical and physiological changes induced by RYGB, new target systems must be investigated[207].

For this purpose, we analyzed for the first time potential changes induced by RYGB on circulating ECs and also the recovery of normal androgens concentrations after this surgery, already shown by some authors[115-117].

It is well known that the ECS is directly involved in food intake and energy balance[11-14]; this relationship has been widely investigated in animals[15-16] while few studies were carried out so far in humans. After the withdrawn from the market of the CB1 antagonist anti-obesity drug Rimonabant[189], it was clear that knoweldge about the role of the ECS in human physiology was lacking. Approximately twenty years ago the first methods were developed to measure ECs in animal tissues[200] with chromatography coupled mass spectrometry. At that time, development of this technique was an epochal change for the emerging field of ECS research. Traditional analytical techniques so far employed were not suitable to accurately measure these molecules, since they are present at very low concentrations and their lipid nature make their isolation and precise quantification even more complicated. This revolutionary technical progress allowed improving new knowledge about ECS.

Chromatography tandem mass spectrometry methods were recently optimized to measure circulating ECs in human plasma[208-209]. During the last decade, several methods were developed and validated, becoming more sensitive and accurate[22-202-203]. So far, three main evidences have been found regarding circulating ECs in humans: first, their levels are higher in obese patients than in normo-weight subjects, suggesting that this up-regulation is related to obesity[17-18]. Second, after diet-induced weight loss, plasma ECs levels are

reduced[21], further confirming the direct relationship between high ECs in plasma and obesity. Third, the EC AEA shows a preprandial rise and a postprandial decrease in plasma, therefore it is likely that this molecule is involved in mechanisms underlying food intake[22]. However the role of plasma ECs is still not clear; some authors hypothesized that since ECs are local mediators, this circulating fraction, widely less abundant than ECs found in tissues, represents a "spill-over" from tissues rather than a biologically active fraction[140].

We therefore designed a project to analyze the involvement of the ECS in weight loss induced by the RYGB. We measured plasma concentrations of AEA, 2AG, PEA and OEA, with a previously developed and validated LC-MS/MS method[22] at the analytical chemistry platform of the Neurocentre Magendie in Bordeaux, in 12 obese patients (BMI > 40 kg/m^2 or BMI > 35 kg/m^2 with obesity associated complications) recruited at the CHU Haut Leveque in Pessac, undergoing a RYGB intervention. Blood withdrawals were performed at three different stages of the study: before the surgery, one month after the surgery and six months after the surgery. To evaluate both basal and dynamic ECs levels, a first blood withdrawal was performed in the morning during fasting state; other blood withdrawals were performed at h. 10:00, 11:00, 11:30. At h. 12:00 patients were exposed to the food and another blood sampling was performed. At h. 12:20 the meal started and further blood withdrawals were performed at the end of the first portion, at the end of the meal and 60, 120 and 180 minutes after the meal.

Our results confirm data reported in literature concerning the efficacy of the RYGB. Our patients lost on average 8,39 ± 0,42% of total initial body weight one month after the surgery and 22,38 ± 1,27% of their total initial body weight six month after the surgery. Several authors reported an average weight loss of approximately 37% of total initial body weight after one year[111]. Considering the percentage of body weight loss reached by our patients six month after the intervention, we can confirm that they are responding very well to the surgery. Similarly, average BMI decreased from a pre-surgery 47,67 ± 1,65 kg/m^2 to 37,22 ± 1,56 kg/m^2 six months after.

Concerning circulating ECs, one month after the surgery we observed a tendency of AEA fasting concentrations to increase, despite the weight loss. Six months after, when patients achieved a further weight loss, AEA fasting levels tended to decrease as compared to measurements carried out one month after the RYGB. In addition, found that plasma AEA changes were statistically significant across the day. We observed also a tendency for plasma AEA to increase right before the meal and decrease after the meal 6 months after RYGB, showing a profile more similar to normal weight subjects. These AEA changes may seem in contrast with the documented ECs decrease after diet-induced weight loss[21]. The different method used to achieve the weight loss may explain this difference: RYGB generates a new anatomy of the GI system in which the stomach is reduced and some parts of the intestine, such as duodenum and part of jejunum[111], are excluded in order to completely avoid contact with nutrients. Evidence from animal studies highlighted an increase of AEA exactly in the duodenum during fasting[164], suggesting a different regulation

of AEA peripheral production during this condition. It is therefore likely that the duodenum exclusion induced by the surgery can be considered as a continuous fasting state, leading to local AEA production.

Another hypothesis that could explain AEA increased concentrations one month after the surgery is an increased lipid mobilization after gastric bypass, which was found by Kullberg and co-workers[210]. In this study, the authors analyzed two groups of patients achieving a similar weight loss with two different strategies: RYGB or life-style modifications. They measured visceral and abdominal subcutaneous fat depots finding an increased percentage of fat loss in the surgery group. Similarly, the percentage of total body fat in the surgery group was lower than in the life-style modifications group[210]. Giving that ECs are derived from lipids, it is likely that the increased lipid mobilization could lead to increased circulating ECs. However, if this was true, all ECs should change in a comparable manner. While, we observed that the RYGB did not lead to significant alterations in 2AG, neither under fasting nor during the meal, confirming results previously published[22].

We found a tendency to decreased PEA concentrations six months after the RYGB. Similarly, a significant difference between in the AUC one and six months after the RYGB was observed. The involvement of PEA in mechanisms underlying food intake and obesity development has never been confirmed; on the other hand, it has been shown that PEA is involved in inflammatory states[176]. It is possible that decrease observed six months after the surgery depends on a decreased inflammatory state. Further analysis, such as the evaluation of inflammatory markers at the three study time-points, will elucidate the observed altered concentration.

On the other hand, several animal studies confirmed the involvement of OEA in food intake[173-175]. Similar to AEA, OEA concentrations increase one month after RYGB; conversely, its plasma levels further rise also six months after the surgery. Furthermore, as well as AEA, OEA presents a pre-prandial peak and a more evident post-prandial decrease after weight loss, six months after the surgery. First studies identified OEA as a potential anorexic molecule, decreasing food intake when injected in animals[173-175]. Our data, on the contrary, shows that OEA may have an opposite role in human plasma, increasing during a fasting condition and decreasing after a meal. Noteworthy, in both the two studies carried out so far investigating OEA in plasma, higher concentrations were found in diabetic over-weight[172] patients and over-weight sleep apnea patients[177]; moreover, in the first study the authors found also an OEA post-prandial decrease[172], consistent with our data.

Probably, OEA has a different regulation and a different role in plasma and in tissues; further studies are required to confirm the role of plasma OEA in food intake regulation in humans.

In conclusion, body weight loss achieved with bariatric surgery may have a different impact on the ECS compared to diet-induced weight loss, likely due to specific anatomic and metabolic changes induced by

the surgery. An accurate analysis of these changes and further studies are required to relate these alterations to important peripheral mechanisms in which the ECS has been shown to have a role.

The relationship between low androgens circulating levels and obesity in men is still not clear. Low testosterone is predictive of visceral obesity development[87] while, on the other hand, it has been clearly shown that visceral obesity leads to hypogonadism and consequently to low androgens circulating levels[10-87]. In the study described in the former chapter, we found highly statistical significant differences between testosterone concentrations in three populations: normo-weight, over-weight and obese, confirming that testosterone levels are inversely proportional to BMI. In this second part of the project, we measured testosterone and DHEA in two male obese patients to test whether normal circulating values of these two steroid hormones are improved after the weight loss surgery-induced. Few studies have been carried out so far to verify the recovery of sexual steroid hormones in men after RYGB. One reason could be the fact that patients undergoing bariatric surgery are mainly women[211]. Data obtained so far are sufficiently consistent to affirm that RYGB induces an increase in androgen plasma concentrations. However, they will need to be further confirmed and strengthened by increasing the number of studied subjects.

We found a significant increase of testosterone plasma levels six months after the surgery, when patients lost approximately 20% of the initial body weight. The same tendency has been confirmed for DHEA, although without statistical significance. At this point, we can confirm what was previously observed by other authors: weight loss induced by RYGB leads to increased androgens circulating levels and possibly to an amelioration of the HPG functionality.

Concerning both ECs and androgens measurements, we found several changes but few of them reached the statistical significance. In this case, the determining factor could be the relative small number of subjects (12 for ECs measurements, 2 for androgen measurements). When recruitment, follow-up and measurements will be completed and we will analyze data from 20 patients, we may have more robust results and therefore provide also a correlation between the circulating levels of these two molecules groups.

Furthermore, we are also recruiting another group of obese subjects who will undergo life-style modifications in order to reach the same body weight loss than our RYGB patients. We will then compare ECs and androgens circulating changes in the two groups of patients to better distinguish the specific role of the surgery in these modifications.

In conclusion, the RYGB is currently one of the most effective anti-obesity therapies, achieving a fast and durable weight loss and generating considerable ameliorations on obesity related co-morbidities. The new anatomy may contribute to the particular effects of this surgery, influencing different biological systems, such as the ECS and the HPG, that are known to play a role in the pathophysiology of obesity. Further studies are required to further characterize the underlying mechanisms.

GENERAL DISCUSSION

The development of LC-MS/MS has been a remarkable turning point in the history of analytical chemistry, allowing the precise identification and quantification of molecules with great improvements in experimental and clinical practice. This technique can be easily introduced in the clinical routine to improve worth and credibility of quantitative tests, essential for accurate diagnoses and follow-up of a high number of pathologies. Similarly, LC-MS/MS may improve different fields of medical research, allowing us to better understand the molecular mechanisms underlying physiological and pathological processes still unclear.

The first study carried out in this thesis has been the comparison between our LC MS/MS method developed for steroids quantification and classic immunoassays. This study put in evidence the already known limits of the traditional techniques. Furthermore, we provided a preliminary redefinition of steroids circulating intervals in healthy subjects. The need to redefine normal ranges is urgent for several classes of molecules, since the intervals currently used in the clinical practice have been defined more than 50 years ago with quantification methods that nowadays show analytical limits. The employment of LC-MS/MS can be precious also to correctly review reference intervals, since the short analysis times allow to easily perform large population screening.

In this thesis this technique has been also used to clarify changes of two important classes of compounds, steroid hormones and ECs, in relation to obesity, currently one of the worldwide relevant health issues. Alterations of these two systems related to obesity are still poorly understood. Especially the precise relationship “cause-effect” is not clear: experimental evidence so far led to highlight a bidirectional link, which must be elucidated. In the study carried out in the first part of the thesis we found significantly lower circulating levels of testosterone in obese patients compared to normo-weight patients. Testosterone can be the classic example of a bidirectional link with obesity: low testosterone is predictive of visceral obesity development and, on the other hand, visceral obesity leads to decreased testosterone levels. In the second part of the thesis we measured testosterone in obese patients before and after a weight loss induced by gastric bypass surgery: six months after the surgery circulating levels of this hormone increase, suggesting that the weight loss is able to restore them. In these patients we evaluated also plasma levels of ECs, lipid molecules circulating at very low concentrations, problematic to measure until the development of LC-MS/MS. Our preliminary data showed a particular effect of the RYGB surgery on the ECS giving us important clues about the modulation of this system in the periphery.

An accurate and trustworthy quantification of these compounds, especially for ECs and steroids circulating at very low concentrations, can lead to better understand the role of these two groups of molecules in the development of obesity or to address future therapeutic strategies. The advantages that this technique may produce for the whole scientific progress were unimaginable only fifty years ago.

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ANNEX

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Serum steroid profiling by isotopic dilution-liquid chromatography–mass spectrometry: Comparison with current immunoassays and reference intervals in healthy adults

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ABSTRACT

Background: The simultaneous, rapid and reliable measurement of a wide steroid panel is a powerful tool to unravel physiological and pathological hormone status. Clinical laboratories are currently dominated by high-throughput immunoassays, but these methods lack specificity due to cross-reactivity and matrix interferences. We developed and validated an isotopic dilution-liquid chromatography–tandem mass spectrometry (ID-LC–MS/MS) method for the simultaneous measurement of cortisol, corticosterone, 11deoxycortisol, androstenedione, deoxycorticosterone (DOC), testosterone, 17OHprogesterone, dehydroepiandrosterone (DHEA) and progesterone in serum, and compared it to routine immunoassays employed in our laboratory. We also established adult reference intervals in 416 healthy subjects.
Methods: 0.9 ml of serum were spiked with labelled internal standards (IS) and extracted on C18 cartridges. Eluate was injected into a two-dimensional LC-system, purified in a perfusion column and separated on a C8 column during a 21 min gradient run. Analytes were revealed by atmospheric pressure chemical ionization (APCI) followed by multiple reaction monitoring (MRM) analysis.
Results: Of the four immunoassays compared with the ID-LC–MS/MS method, only the results of ElecsysE170 for cortisol, testosterone in males and progesterone > 1 ng/ml were in agreement with ID-LC–MS/MS. ElecsysE170 for testosterone in females and progesterone < 1 ng/ml, Immulite2000 for androstenedione, DSL-9000 for DHEA and 17OHP Bridge for 17OHprogesterone, respectively, showed poor agreement. Reference intervals and steroid age and fertility related fluctuations were established.
Conclusion: Our ID-LC–MS/MS method proved to be reliable and sensitive in revealing steroid circulating concentrations in adults and in highlighting the limits of routine immunoassays at low concentrations.

1. Introduction

Steroid measurement remains a challenge for the endocrinological community. Since the RIA breakthrough 40 years ago [1,2], immunoassays have remained the most common tool to characterize the pathophysiological states of circulating steroids. RIA combined with extraction and chromatographic purification steps have been attributed acceptable specificity and good sensitivity. Nowadays, most methodologies are dominated by automated chemiluminescent or electro-chemiluminescent immunoassay platforms and by semi-automated RIAs. These assays offer simplicity and high throughput, key-factors for a large routine application, but due to cross-reactivity and matrix interferences they lack specificity [3]. Poor validation and standardization data are often provided for these methods, causing a huge variability among dif-

Abbreviations: ID-LC–MS/MS, isotopic dilution-liquid chromatography–tandem mass spectrometry; DOC, deoxycorticosterone; DHEA, dehydroepiandrosterone; IS, internal standard; APCI, atmospheric pressure chemical ionization; MRM, multiple reaction monitoring; GC–MS, gas chromatography–mass spectrometry; DHEA-S, DHEA-sulphate; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; SPE, solid phase extraction; QC, quality control; BMI, body mass index; CAD, collision activated dissociation; CUR, curtain gas; LLOQ, lower limit of quantification; S/N, signal to noise ratio; LOD, limit of detection; IR, ion ratio; IQR, interquartile range; CI, confidence interval; MW, molecular weight; RT, retention time; DP, declustering potential; CE, collision energy; CXP, cell exit potential; $S_{y|x}$, standard deviation of residuals; M, males; F, females; pre-M, pre-menopausal females; post-M, post-menopausal females; s.d., standard deviation.

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ferent kits and among different laboratories employing the same kits and leading to significant variations in reference intervals from one commercial assay to another [4,5]. These drawbacks limit diagnostic accuracy, appropriate treatment and follow-up in clinical praxis, and preclude epidemiological multicenter studies [6–9].

Gas chromatography–mass spectrometry (GC–MS) was first introduced in the 1960s and improved in the 1980s to represent the gold standard method for steroid analysis [10–12]. However, this technique requires complex time-consuming derivatization procedures for sample pre-treatment, limiting its application in routine clinical practice. Conversely, ID-LC–MS/MS is an innovative technology combining the high selectivity and sensitivity of mass spectrometry with the versatility of liquid chromatography. ID-LC–MS/MS allows reliable, simultaneous quantification of a wide panel of steroids in a broad concentration range with high throughput capabilities [13]. These advantages have yielded insights into biochemical changes and more useful clinical data, but a re-definition of age and sex specific reference intervals is urgently needed. Many ID-LC–MS/MS methods for steroid measurement have been proposed in recent years [14–22], often performing multi-hormone analysis in a short chromatographic run and requiring less-demanding sample preparation. However, a good pre-analytical treatment and a careful chromatographic separation of frequently occurring isobaric analytes are still needed for sensitive and specific steroid monitoring in complex matrices like serum or urine [23].

This study describes the development and validation of an ID-LC–MS/MS method for the simultaneous measurement of nine serum steroids of clinical relevance, comparing it with six routinely used immunoassays. In addition, we evaluated the steroid profile of 416 healthy normal weight drug-free subjects, aged 18–89 years, analyzing the influence of age and fertility status on steroid levels.

2. Materials and methods

2.1. Chemicals

The following compounds were used: cortisol, corticosterone, 11deoxycortisol, androstenedione, DOC, testosterone, 17OHprogesterone, DHEA, progesterone, 21deoxycortisol, epitestosterone, DHEA-sulphate (DHEA-S) and cortisone (Steraloids, Newport, RI); d4-cortisol, d8-corticosterone, d2-11deoxycortisol, d5-testosterone, d8-17OHprogesterone, d2-DHEA and d9-progesterone (CDN Isotopes, Pointe Claire, Canada); ^{13}C2-testosterone (Cambridge Isotope Laboratories, Andover, MA); bovine serum albumin (BSA), prednisone and prednisolone (Sigma–Aldrich, St. Louis, MO); betamethasone disodium phosphate (Defiante Farmaceutica, Madeira, Portugal); dexamethasone 21phosphate disodium salt (Visufarma, Rome, Italy); methylprednisolone acetate (Pfizer, New York City, NY); triamcinolone acetonide (Bristol-Myers Squibb, New York City, NY). Gradient grade methanol and zinc sulphate hepta-hydrated were from Merck (Darmstadt, Germany); ultrapure water was produced by MilliQ Gradient A10 system (Millipore, Volketswil, Switzerland). Steroid-free serum was from MP Biomedicals (Solon, OH). The solid phase extraction (SPE) cartridges were IST Isolute C18 100 mg, 1 cm^3 from Biotage (Uppsala, Sweden). Reference material was from the Reference Institute for Bioanalytics (Bonn, Germany).

2.2. Standard solutions, calibrators and in-house quality control (QC) samples

Stock solutions were prepared in methanol for each standard and isotopically labelled IS at different concentrations in the mg/ml range. Working solutions were at 100 ug/ml for cortisol, progesterone and ISs, and at 10 ug/ml for other analytes. A stock calibrator was prepared by mixing each standard to obtain the following concentrations: cortisol, 500 ng/ml; DHEA and progesterone, 50 ng/ml; corticosterone, androstenedione and testosterone, 20 ng/ml; 11deoxycortisol, DOC and 17OHprogesterone, 10 ng/ml. An eight-point calibration curve was prepared by serial dilution of the stock calibrator in 4% BSA. The BSA solution represented the "zero" calibration point. The working IS solution was a mixture of d4-cortisol 50 ng/ml, d8-corticosterone and d2-11deoxycortisol 5 ng/ml, d2-DHEA 3 ng/ml, ^{13}C2-testosterone 2 ng/ml and d8-17OHprogesterone 1 ng/ml, d9-progesterone 10 ng/ml. Stock solutions, working solutions and calibrators were stored at −20 °C. Three in-house QCs were prepared by generating a serum pool used as the low level and by adding standard solutions to obtain the medium and high levels for each analyte. Calibrators for androstenedione determination at 0.5 and 5 ng/ml of Immulite2000 (Siemens Healthcare Diagnostics, Deerfield, IL), at 0.98, 2.5, 9.8 and 25.0 ng/ml of DSL-9000 RIA kit (Webster, TX) and at 0.1, 0.35, 2.0 and 10.0 ng/ml of 17OHP Bridge RIA kit (Adaltis, Guidonia, Italy) were measured by ID-LC–MS/MS as such for calibration assessment.

2.3. Specimens

De-identified samples for method comparison were collected among sera from S.Orsola-Malpighi Hospital routine laboratory. Healthy volunteers, males and females aged 19–89 years, were recruited for reference interval estimation, after having given their informed consent, at the local health service of the town of Massa Lombarda. The study was approved by the local Ethical Committee. Body mass index (BMI) ranged between 18.1 and 25.0 kg/m^2. The inclusion criteria were: body weight stability in the last 3 months, complete sexual development and menstrual cycle regularity in fertile women. Subjects taking drugs (except for antipyretic or anti-inflammatory compounds), or presenting endocrine, hepatic, renal, tumoral, autoimmune, cardiovascular, hematologic, neurologic or psychiatric diseases, sleep disorders, or allergies requiring treatment were excluded. Between 8 and 10 a.m., subjects were infused with saline for 10 min before blood collection in a Vacuette Z serum beads clot activator (Greiner Bio-One, Kremsmunster, Austria); samples were centrifuged at 2000 × *g* for 10 min at room temperature and sera were stored in 1.5 ml polypropylene tubes at −20 °C until analysis.

2.4. Sample preparation

Serum samples and in-house QCs were thawed and vortexed. Liophilic reference samples were reconstituted with 3 ml H_2O and gently mixed at room temperature for 60 min. For each sample, curve calibrator, immunoassay calibrators, and QCs 900 ul were transferred to 12 mm × 75 mm glass tubes before addition of 1 ml of MeOH:[$(ZnSO_4)(H_2O)_7$ aqueous solution 8.9%,w/v] = 80:20 containing IS. After 3 min vortex, tubes were centrifuged for 10 min at 2000 × *g* at room temperature. Supernatants were transferred on the SPE cartridge previously activated with 1 ml of MeOH and conditioned with 1 ml of H_2O. After washing with 3 ml of H_2O cartridges were eluted with 1 ml of MeOH. Eluates were dried under nitrogen stream, reconstituted with 0.3 ml of 50% MeOH and transferred into glass vials and placed into a Series 200 Autosampler thermostated at 10 °C (PerkinElmer, Waltham, MA).

2.5. On-line purification and LC separation

Two hundred microliters were injected into a two-dimensional LC-system consisting in a modular HPLC Series 200 by PerkinElmer, further purified on perfusion column POROS

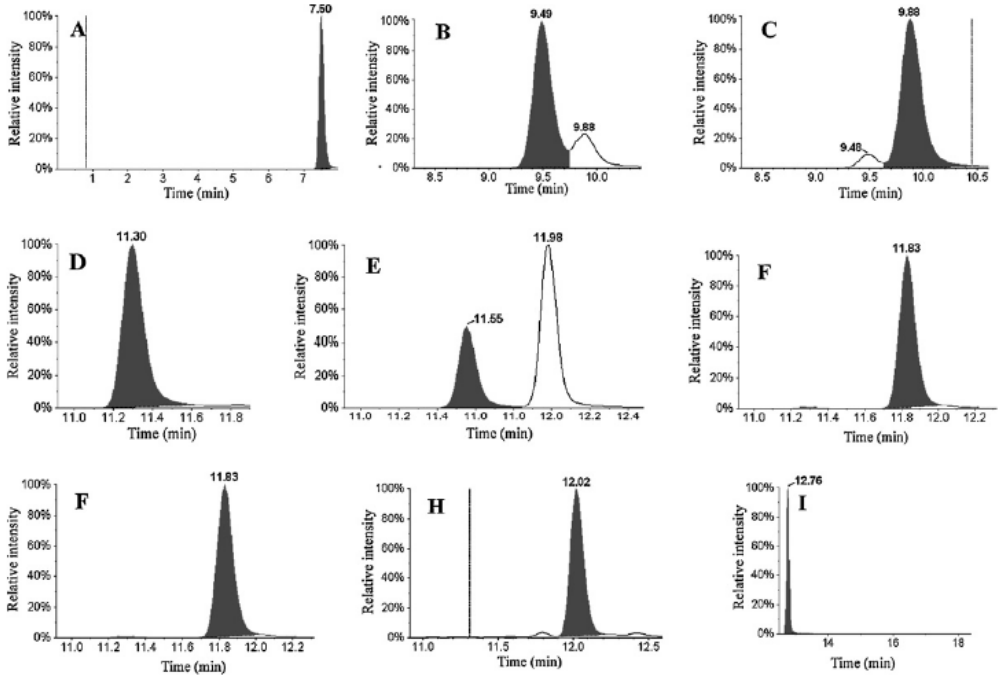

Fig. 1. Chromatographic peak with its retention time for cortisol (A), corticosterone (B), 11deoxycortisol (C), androstenedione (D), DOC (E), testosterone (F), 17OHprogesterone (G), DHEA (H) and progesterone (I).

R1/20 2.1 mm × 30 mm by Applied Biosystems (Foster City, CA). After washing with 10% MeOH at 3 ml/min for 1 min, the sample was back-flushed to the analytical column Luna RP-C8 100 mm × 4.6 mm, 5 um (Phenomenex, Torrance, CA) equipped with an RP-C8 4 mm × 2 mm, 5 um guard column, through a ten-port switching valve (VICI, Houston, TX) at 0.750 ml/min of an eluent made with 98% solvent A (20% MeOH) and 2% solvent B (100% MeOH). The 21 min gradient run program started with 45% solvent B and at min 8.5 a linear gradient to 100% in 2 min was activated with a subsequent 2 min washing step and a 6.5 min re-equilibration step to the initial conditions. Chromatographic peaks are shown in Fig. 1. Chromatographic resolution was achieved for the isobaric steroid pairs (corticosterone and 11deoxycortisol, DOC and 17OHprogesterone, testosterone and DHEA) and for the steroids, like testosterone and androstenedione, which differ in their molecular weight by only 2 amu and which could interfere through the specific +2 amu-isotopomer of one analyte over the mono-isotopic form of the other.

2.6. Mass spectrometry detection

Mass spectrometric measurements were performed by an API 4000-QTrap (AB-Sciex, Toronto, Canada) working in triple-quadrupole mode. Quantification was performed by the MRM mode, choosing for each analyte two specific transitions, one for the quantitative assessment (the "quantifier") and one for confirmation (the "qualifier"). The parameters pertaining to the MRM transitions were optimized by infusing standard solutions at concentrations ranging from 100 ng/ml to 10 ug/ml, into the Turbo-V source through an infusion pump set at 10 ul/min in addition to a makeup flow of 50% MeOH at 400 ul/min (Table 1). The APCI probe operated with a Corona discharge current of 3 uA in positive ion mode. Collision activated dissociation (CAD) gas was nitrogen set at a pressure of 10 mTorr and the other parameter settings were: probe temperature 400 °C, curtain gas (CUR) 30 psi, nebulising gas 30 psi. To maximize the dwell time for each targeted analyte, LC-run monitoring was divided into four periods: 0.0–8.3 min; 8.4–10.9 min; 11.0–12.5 min; 12.6–21.0 min. Unit mass resolution was set at both Q1 and Q3.

2.7. Quantitation

Data processing and quantitation were performed by Analyst 1.4.2 software package by AB-Sciex. Calibration was done through linear regression: concentrations for each analyte were back calculated by interpolation on the respective regression curve.

2.8. ID-LC–MS/MS method validation

Four per cent BSA was chosen as a suitable matrix for standard calibration points. The isotopic dilution quantitation method was assessed as follows: d4-cortisol was used as IS for cortisol, d8-corticosterone for corticosterone; d2-11deoxycortisol for 11deoxycortisol; ^{13}C2-testosterone for androstenedione, testosterone and DHEA; d8-17OHprogesterone for DOC, 17OHprogesterone and progesterone. D5-testosterone, d2-DHEA and d9-progesterone were discarded since their unsuitability for the remarkable signal instability with the APCI source (likely more than on the electrospray source), and for the cross-interference on the unlabelled standard steroid transitions; analogous phenomena were also reported by Vogeser and co-workers in a recent publication [24]. A $1/x$ weighting regression was chosen to ensure higher accuracy and precision at the low concentration end of the curve. Linearity was achieved for three-four orders of magnitude. The lower limit of quantification (LLOQ) was determined on five replicates as the lowest concentration exhibiting a signal to noise ratio (S/N) above ten, with an accuracy between 80 and 120% of the true value and with CV below 20%. The limit of detection (LOD) was determined as the lowest

Table 1
Experimental conditions for the ID-LC–APCI-MS/MS detection for each steroid and IS: molecular weight (MW), measurement period, retention time (RT, min), precursor ion (Q1 mass, *m/z*), fragment ion (Q3 mass, *m/z*), declustering potential (DP, V), collision energy (CE, eV), cell exit potential (CXP, V), and observed IR are reported for each targeted compound.

Analyte	MW	Period	RT	Transition	Q1 mass	Q3 mass	DP	CE	CXP	IR
Cortisol	362.46	1	7.4	Quantifier	363.2	121.2	60	45	3	4.3
				Qualifier	363.2	267.4	60	35	5	
D4-cortisol	366.46	1	7.4	IS	367.3	97.1	50	45	3	
Corticosterone	346.46	2	9.5	Quantifier	347.1	121.0	76	45	9	2.1
				Qualifier	347.1	97.1	76	45	5	
D8-corticosterone	354.46	2	9.4	IS	355.4	125.4	88	45	5	
11Deoxycortisol	346.46	2	9.9	Quantifier	347.2	109.1	82	45	5	1.1
				Qualifier	347.2	97.0	82	45	5	
D2-11deoxycortisol	348.46	2	9.9	IS	349.4	97.1	75	45	3	
Androstenedione	286.41	3	11.3	Quantifier	287.4	97.0	78	30	3	1.4
				Qualifier	287.4	109.0	78	40	5	
DOC	330.5	3	11.6	Quantifier	331.4	109.1	80	40	4	1.1
				Qualifier	331.4	97.0	80	30	4	
Testosterone	288.42	3	11.8	Quantifier	289.2	97.1	78	35	3	1.0
				Qualifier	289.2	109.1	78	35	5	
^{13}C2-testosterone	290.41	3	11.8	IS	291.4	111.1	74	35	5	
17OHProgesterone	330.46	3	12.0	Quantifier	331.1	97.0	70	40	3	0.9
				Qualifier	331.1	109.3	70	45	5	
D8-17OHProgesterone	338.46	3	11.9	IS	339.5	100.1	50	45	7	
DHEA	288.42	3	12.1	Quantifier	271.3	197.2	55	25	3	3.0
				Qualifier	271.3	213.3	55	25	4	
Progesterone	314.46	4	12.8	Quantifier	315.6	97.1	80	30	3	1.1
				Qualifier	315.6	109.1	80	40	4	

concentration exhibiting a S/N above three. Data are summarized in Table 2.

Interference with other endogenous and exogenous steroids was investigated. Amounts of 21deoxycortisol, epitestosterone, DHEA-S, cortisone, prednisone and prednisolone were injected into the LC–MS/MS system: traces shown in Supplemental Fig. 1 highlight the good immunity of the monitored steroids from the massive presence of the others. Triamcinolone acetonide, methylprednisolone, dexamethasone and betamethasone, were spiked at 500 ng/ml into the QC samples and processed as unknown samples: no interference over calculated concentrations of monitored steroids was found. No interference by serum matrix non-steroid compounds was shown by injecting steroid-free serum samples. Intensity ratios between quantifier and qualifier transitions (ion ratio, IR) were monitored in each sample to check for any unexpected interference. Sample IR was accepted within 20% of the calibrator IR.

Method imprecision was assessed on six replicates per day of in-house QC samples at low, medium and high concentrations (intra-assay), and on six different days (inter-assay). CV was accepted below 15% in the intra-assay and below 20% in inter-assay.

Trueness, expressed as the percentage of found concentration over nominal concentration, was assessed in the low, medium and high range by reference certified sera for cortisol, testosterone, 17OHprogesterone and progesterone, and by in-house validation for corticosterone, 11deoxycortisol, androstenedione, DOC and DHEA, by spiking gravimetrically determined quantities of pure standards in steroid-free serum, previously checked for absence of steroids above the LOD. Data are summarized in Table 3.

Sensitivity was also evaluated in serum matrix, as reported for LLOQ, by spiking minimal amounts of analyte standards in steroid-free serum in three replicates (Table 2).

Ion suppression was investigated by spiking equal amounts of standard analytes either on pre-extracted BSA solutions or steroid-free serum, to exclude any procedural losses from the yield calculation. Analyte peak areas were compared in BSA and in steroid-free serum, and both were compared to peak areas of pure standards (Supplemental Table 1). Negligible deviation from 100%, denoting absence of suppression, was observed. A post-column infusion of a mixture containing the nine steroids at concentrations suitable for generating measurable steady-state signals was performed during injections of blank and steroid-free serum

Table 2
ID-LC–MS/MS method calibration curve and sensitivity ($S_{y/x}$: standard deviation of residuals).

	Linear range (ng/ml)	Slope	Intercept	$S_{y/x}$	r^2	LLOQ				LOD (pg on column)	Sensitivity in serum matrix (ng/ml)
						ng/ml	S/N	CV %	Accuracy %		
Cortisol	0.244–500.0	0.0551 ± 0.0022	0.0050 ± 0.0022	1.673	0.9997	0.2440	49	9.0	99.3	4.8	0.244
Corticosterone	0.039–20.0	0.2652 ± 0.0213	−0.0003 ± 0.0019	0.100	0.9995	0.0391	13	3.4	95.9	4.0	0.313
11Deoxycortisol	0.019–10.0	0.1748 ± 0.0106	0.0025 ± 0.0014	0.075	0.9993	0.0195	14	15.3	94.1	2.0	0.078
Androstenedione	0.019–20.0	0.4577 ± 0.0139	0.0032 ± 0.0013	0.047	0.9998	0.0195	14	13.2	100.7	2.3	0.039
DOC	0.019–10.0	0.8857 ± 0.0451	−0.0006 ± 0.0018	0.032	0.9994	0.0195	11	9.6	107.8	2.5	0.078
Testosterone	0.019–20.0	0.4922 ± 0.0145	0.0158 ± 0.0027	0.110	0.9993	0.0195	11	6.2	94.2	2.8	0.019
17OHProgesterone	0.010–10.0	0.9012 ± 0.0649	0.0011 ± 0.0023	0.033	0.9996	0.0098	11	8.9	107.5	1.7	0.078
DHEA	0.195–50.0	0.0164 ± 0.0036	0.0002 ± 0.0001	0.348	0.9995	0.1953	10	11.2	97.4	29.2	0.781
Progesterone	0.024–50.0	0.4527 ± 0.0273	0.0035 ± 0.0024	0.117	0.9999	0.0244	29	9.3	103.4	1.2	0.049

Table 3
ID-LC–MS/MS method trueness and imprecision at low, medium and high range.

	Low range					Medium range					High range				
	ng/ml	Trueness %	ng/ml	Intra-assay CV %	Inter-assay CV %	ng/ml	Trueness %	ng/ml	Intra-assay CV %	Inter-assay CV %	ng/ml	Trueness %	ng/ml	Intra-assay CV %	Inter-assay CV %
Cortisol	63.7	94.9	25.4	3	8	129.0	103.7	144.8	3	7	446.2	93.6	415.1	2	5
Corticosterone	1.00	92.5	1.12	2	10	5.00	98.3	5.41	6	5	15.00	98.5	16.25	3	7
11Deoxycortisol	0.400	106.2	0.260	8	8	2.000	99.1	0.782	4	5	8.000	102.3	4.407	3	2
Androstenedione	0.400	86.3	0.291	10	11	5.000	97.9	4.239	10	10	15.000	100.6	17.810	7	11
DOC	0.080	100.7	0.114	5	6	1.000	100.0	0.702	6	9	8.000	104.1	7.783	6	9
Testosterone	0.300	99.9	0.299	4	7	2.300	97.4	2.218	4	7	8.800	99.6	13.126	3	4
17OHProgesterone	0.529	104.4	0.696	5	9	2.430	102.1	4.562	4	5	7.534	101.4	9.810	4	5
DHEA	1.00	94.6	2.05	8	8	15.00	101.8	14.00	7	9	40.00	98.2	43.64	8	10
Progesterone	0.842	83.7	0.129	8	11	4.214	92.4	5.369	5	6	29.340	92.0	23.123	5	6

extracts. Negligible increase or decrease in MRM scan intensities were observed, denoting a negligible ion suppression effect.

2.9. Immunoassays

Cortisol, testosterone and progesterone were measured by electro-chemiluminescence immunoassay on the Modular Analytics ElecsysE170 by Roche Diagnostics (Mannheim, Germany); androstenedione by the solid-phase, competitive chemiluminescent enzyme immunoassay Immulite2000; DHEA by DSL-9000 RIA and 17OHprogesterone by 17OHP Bridge RIA.

2.10. Data analysis and statistics

2.10.1. Method comparison

ID-LC–MS/MS method was compared with the immunoassay measurements for cortisol (n = 159), testosterone (n = 162) progesterone (n = 85), androstenedione (n = 137), DHEA (n = 143) and 17OHprogesterone (n = 99). To avoid bias attributable to different sensitivity limits, results below the sensitivity limit were excluded. Calibration agreement between Immulite2000, DSL-9000 and 17OHP Bridge and ID-LC–MS/MS was assessed by measuring kit calibrators by ID-LC–MS/MS. No comparison was performed for corticosterone, 11deoxycortisol and DOC since no routine methods were available in the reference laboratory.

Non-normally distributed variables were compared by the Mann–Whitney test and all data are expressed as median and interquartile range (IQR). The Deming regression was applied to account for the imprecision of both methods [25]. Bland and Altman plots were drawn for agreement estimation, representing the percentage difference between the methods against the mean [26].

2.10.2. Estimation of reference intervals

Median values and non-parametric 2.5th and 97.5th centiles [27] were estimated in males (n = 217) and females (n = 199). The female group was subdivided according to fertility status into premenopausal (n = 134 of whom 51 women in the follicular phase [days 1–10]), and post-menopausal (n = 65) subgroups. Reference intervals in luteal phase were not evaluated because of the small number of samples available. The effect of age on steroid level was evaluated by the Spearman regression analysis.

Data analysis was performed on MedCalc v9.3.7.0 (Mariakerke, Belgium).

3. Results

3.1. Method comparison study

By the Mann–Whitney comparison, ElecsysE170 and ID-LC–MS/MS methods provided non-different results for testosterone in males and for progesterone above 1 ng/ml. Lower median values were obtained by ID-LC–MS/MS compared with ElecsysE170 for determination of cortisol (−16%, p = 0.0052), testosterone in females (−26%, p = 0.0080) and progesterone below 1 ng/ml (−84%, p < 0.0001). ID-LC–MS/MS, compared to Immulite2000, DSL-9000 and 17OHP Bridge for measurement of androstenedione, DHEA and 17OHprogesterone, respectively, provided significantly lower results (p < 0.0001), immunoassay medians being 2–3-fold higher than ID-LC–MS/MS medians (Supplemental Table 2).

Deming regression graphs, slope and intercept coefficients with respective standard errors and correlation coefficient between immunoassays and ID-LC–MS/MS are reported in Fig. 2. In males, the slope and the intercept obtained from the regression between testosterone results by ElecsysE170 and ID-LC–MS/MS were not different from one and zero, parameters of the curve of best

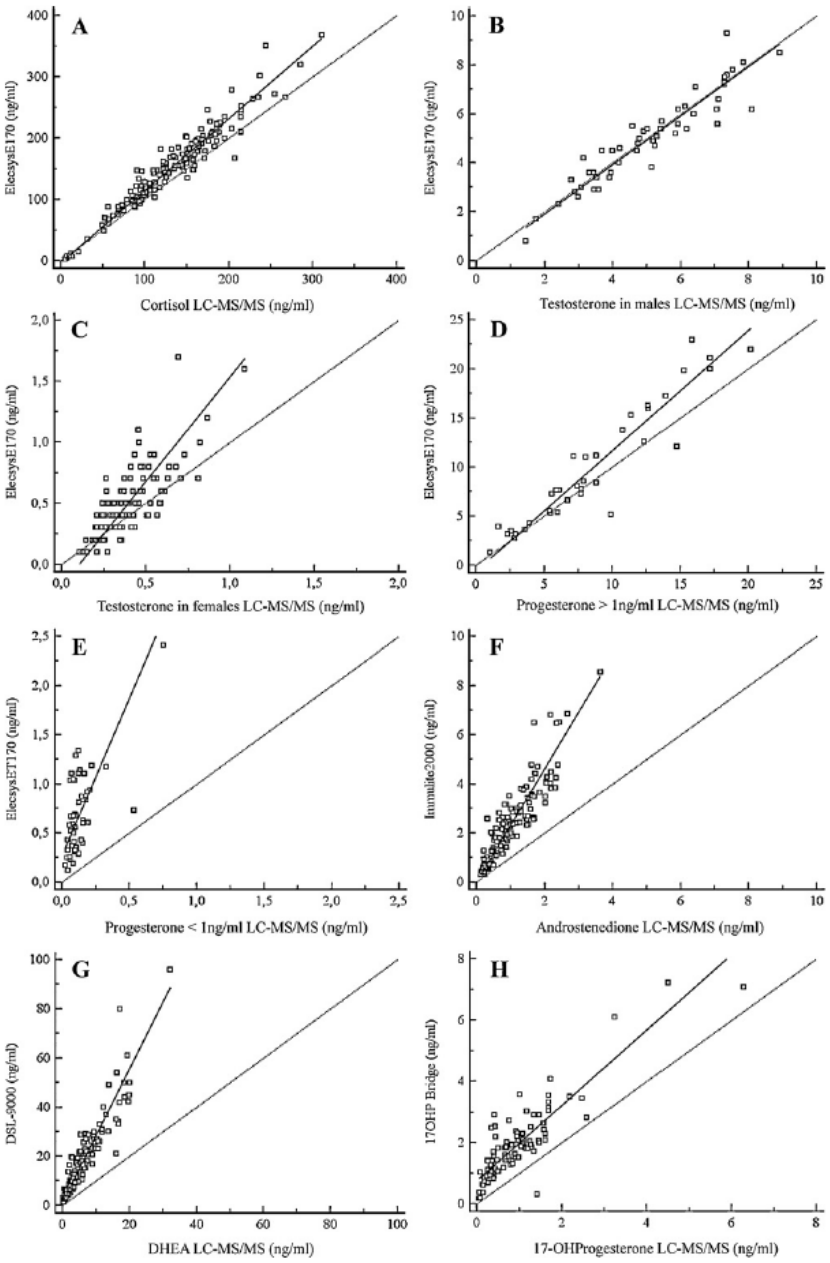

Fig. 2. Deming regression line (black) and line of best fit (dotted) for cortisol (A: $m^* = 1.176 \pm 0.029$; $q^\# = -3.093 \pm 3.147$; $r = 0.968$), testosterone in males (B: $m^* = 1.004 \pm 0.062$; $q^\# = -0.097 \pm 0.283$; $r = 0.938$), testosterone in females (C: $m^* = 1.724 \pm 0.208$; $q^\# = -0.181 \pm 0.073$; $r = 0.773$), progesterone > 1 ng/ml (D: $m^* = 1.227 \pm 0.074$; $q^\# = -0.587 \pm 0.573$; $r = 0.946$), progesterone < 1 ng/ml (E: $m^* = 3.201 \pm 1.466$; $q^\# = 0.270 \pm 0.166$; $r = 0.637$), androstenedione (F: $m^* = 2.299 \pm 0.117$; $q^\# = 0.042 \pm 0.093$; $r = 0.906$), DHEA (G: $m^* = 2.708 \pm 0.182$; $q^\# = 1.478 \pm 0.800$; $r = 0.929$) and 17OHprogesteorne (H: $m^* = 1.230 \pm 0.181$; $q^\# = 0.769 \pm 0.152$; $r = 0.874$); m^* = slope; $q^\#$ = intercept.

fit, respectively. A slight but statistically elevated slope and an intercept not different from zero were obtained for cortisol and progesterone above 1 ng/ml [95% confidence interval (CI): 1.119–1.233 and 1.077–1.378, respectively].

Testosterone in females and progesterone below 1 ng/ml exhibited elevated slope coefficients (95%CI: 1.311–2.137 and 0.253–6.149, respectively) and testosterone in females had a negative intercept coefficient (95%CI: −0.326 to −0.036). The regression between ID-LC–MS/MS and Immulite2000, DSL-9000 and 17OHP Bridge, for the determination of androstenedione, DHEA and 17OHprogesterone provided significantly elevated slopes (95%CI: 2.068–2.530; 2.348–3.068; 0.870–1.589, respectively), but only 17OHprogesterone regression provided a non-negligible positive intercept coefficient (95%CI: 0.468–1.071).

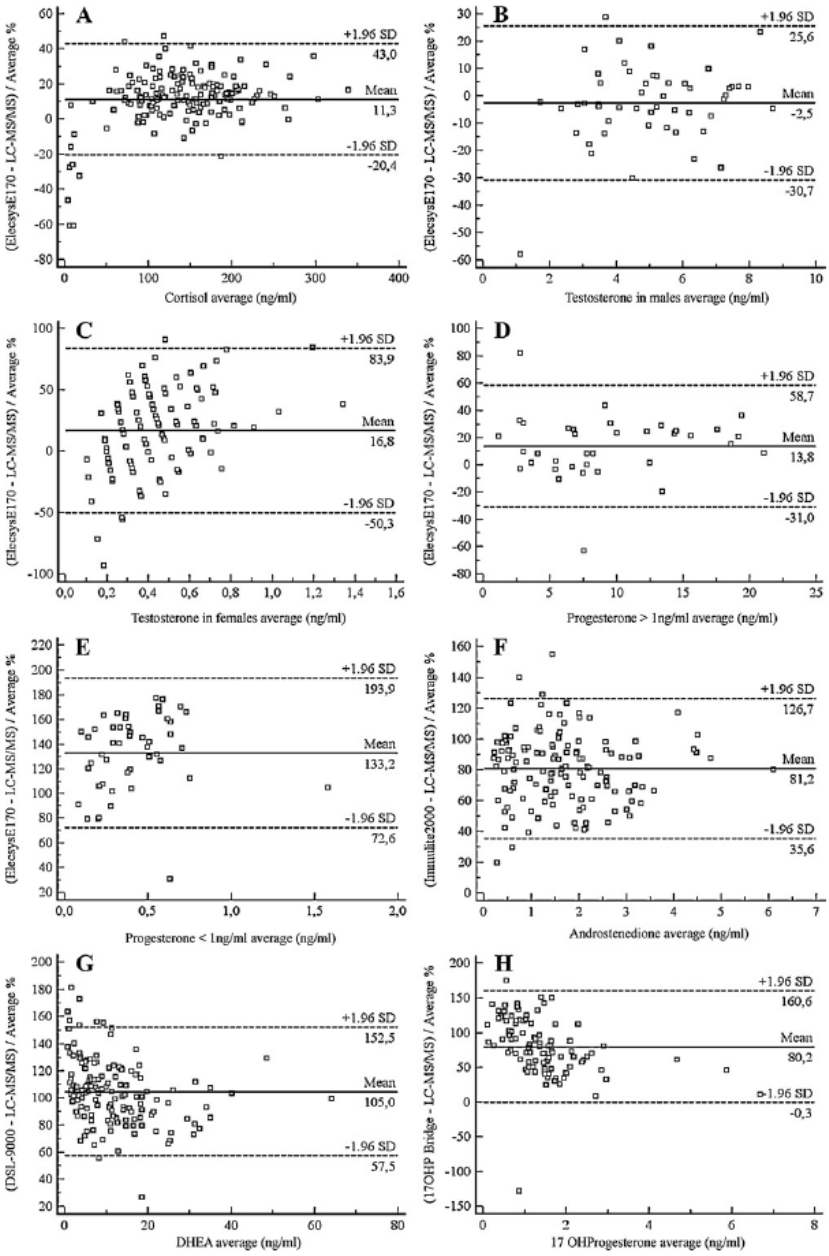

Fig. 3. Bland and Altman plot of cortisol (A), testosterone in males (B), testosterone in females (C), progesterone > 1 ng/ml (D), progesterone < 1 ng/ml (E), androstenedione (F), DHEA (G), 17OHprogesterone (H); *y*-axis: percentage difference between results by immunoassay and LC–MS/MS on average; *x*-axis: average results of the two methods.

By the Bland and Altman agreement estimation we found that among the four immunoassays compared to ID-LC–MS/MS method, only ElecsysE170 method for all analytes, except progesterone below 1 ng/ml, provided an acceptable mean difference. However, only for testosterone in males the mean difference was not different from 0% (95%CI: −6.6% to 1.5%). For all other analytes worse agreement was obtained, with mean differences ranging from 80.2% of 17OHprogesterone to 133.2% of progesterone below 1 ng/ml. Furthermore, despite the notable width, agreement intervals for androstenedione, DHEA, 17OHprogesterone and progesterone below 1 ng/ml were entirely located on the positive side of the graph, with only one case in which measurement of 17OHprogesterone was higher for immunoassay compared to ID-LC–MS/MS (Fig. 3). These data indicated a good accuracy and calibration of ElecsysE170 for the determination of cortisol, testosterone and progesterone, analytes for which trueness of the ID-LC–MS/MS method was confirmed against GC–MS certified sera. Nevertheless, Deming regression, correlation of coefficients and Bland and Altman analysis showed that ElecsysE170 is affected by cross-reactivity at low ranges of testosterone and progesterone.

Table 4
Steroid median concentrations and 2.5–97.5th percentiles (ng/ml) in adult males (M) and pre-menopausal (pre-M), follicular and post-menopausal (post-M) females (F) (s.d.: standard deviation). Blood samples were taken from 8:00 to 10:00 a.m.

	M	F pre-M	F follicular	F post-M
Age	18–89	18–54	18–54	45–86
BMI (mean ± s.d.)	23.2 ± 1.7	21.7 ± 2.0	21.8 ± 2.0	23.2 ± 1.7
N	217	134	51	65
Hormone	Median (2.5–97.5P)	Median (2.5–9 7.5P)	Median (2.5–97.5P)	Median (2.5–97.5P)
Cortisol	119.9 (45.7–199.4)	101.5 (47.4–199.7)	113.1 (40.5–199.8)	114.7 (56.9–180.4)
Corticosterone	3.23 (0.46–12.60)	2.62 (0.62–11.85)	2.89 (0.45–11.94)	2.74 (0.68–8.54)
11Deoxycortisol	0.325 (0.086–1.094)	0.239 (<1.081)	0.249 (<1.345)	0.284 (0.082–0.838)
Androstenedione	0.571 (0.262–1.263)	0.748 (0.277–1.638)	0.727 (0.308–1.602)	0.299 (0.095–0.773)
Testosterone	5.34 (2.82–8.18)	0.248 (0.104–0.454)	0.248 (0.116–0.431)	0.147 (0.077–0.392)
DHEA	4.97 (1.40–14.28)	5.09 (1.19–18.93)	5.68 (2.03–27.04)	2.45 (0.80–6.60)
17OHProgesterone	1.095 (0.415–2.542)	0.578 (0.152–2.266)	0.411 (0.161–0.947)	0.209 (<0.527)
Progesterone	0.078 (<0.189)	0.203 (<17.816)	0.093 (<1.673)	< 0.049 (<0.080)

The results given by the measurement of 17OHP Bridge calibrators by ID-LC–MS/MS assessed the good calibration of this kit, trueness of the ID-LC–MS/MS method being confirmed against GC–MS certified sera: accuracy at points 0.1, 0.35, 2.0 and 10.0 ng/ml was 92.8%, 101.3%, 96.7% and 101.6%, respectively. The data above, together with the poor correlation coefficient (Fig. 2), the discrepancies observed in the Mann–Whitney comparison (Supplemental Table 2) and the results obtained by the Bland and Altman analysis (Figs. 2 and 3), clearly explain that the overestimation by 17OHP Bridge RIA is due to the cross-reactivity and not to the miscalibration. By measuring DHEA in DSL-9000 calibrators by ID-LC–MS/MS, we found an accuracy of 53.9%, 48.5%, 46.2% and 55.5% at points 0.98, 2.5, 9.8 and 25 ng/ml, respectively. Such a miscalibration can only in part explain the huge overestimation shown by the Mann–Whitney comparison and by the regression analysis. The wide agreement range (57.5–152.5%) obtained in the Bland and Altman plots suggested the presence of a non-proportional bias, probably due to the cross-reactivity of the antibody employed in the assay. The accuracy obtained by the measurement of androstenedione in Immulite2000 calibrators was 133.1% and 82.5% at 0.5 and at 5.0 ng/ml, respectively. Similarly to DSL-9000, this miscalibration may explain only part of the overestimation exhibited by Immulite2000 (Fig. 1, Supplemental Table 2), but both the 2.5-fold increase observed in the Mann–Whitney comparison and the wide range of agreement observed in the Bland and Altman analysis (35.6–126.7%) suggest a further contribute due to cross-reactivity (Fig. 3).

3.2. Reference intervals

Reference intervals and median values are listed in Table 4. The reference interval for DOC was not set because, despite a LLOQ of 19.5 pg/ml, the sensitivity in serum matrix (78 pg/ml) proved unsatisfactory for the measurement of this hormone. A single measurement of corticosterone, testosterone and 17OHprogesterone, two of DHEA, four of 11deoxycortisol and 28 of progesterone were discarded because of the questionable IRs.

In females, a single measurement of corticosterone, androstenedione and DHEA, 13 of 11deoxycortisol (8% of pre-menopausal and 3% of post-menopausal), three of 17OHprogesterone and 65 of progesterone, 46 of them in the post-menopausal subgroup (71%), whereas in males four measurements of 11deoxycortisol, and 39 of progesterone were below the sensitivity limit.

In males, androstenedione, testosterone, DHEA, 17OHprogesterone and progesterone exhibited a significant negative correlation with age (Supplemental Fig. 2) [rho = −0.236 ($p = 0.0005$); −0.224 ($p = 0.0010$); −0.576 ($p < 0.0001$); −0.185 ($p = 0.0066$) and −0.268 ($p = 0.0002$), respectively]. In the pre-menopausal subgroup an age-dependent decrease of androstenedione, testosterone and DHEA was also observed (Supplemental Fig. 3) [rho = −0.334 ($p = 0.0001$); −0.232 ($p = 0.0076$) and −0.466 ($p < 0.0001$), respectively] and it continued for DHEA, but not for androstenedione and testosterone, in the post-menopausal subgroup (rho = −0.282, $p = 0.024$), whereas an age-dependent increase was observed for cortisol and 11deoxycortisol: rho = 0.369 ($p = 0.0031$) and 0.401 ($p = 0.0015$), respectively. In the post-menopausal subgroup, levels of androstenedione, testosterone and DHEA were significantly lower than pre-menopausal levels, and levels of 17OHprogesterone and progesterone lower than follicular phase levels ($p < 0.0001$ for all). No significant changes were observed for cortisol and 11deoxycortisol.

4. Discussion

We developed a sensitive ID-LC–MS/MS method for the simultaneous measurement of nine serum steroids. This is a powerful tool in clinical praxis to depict various pathophysiological alterations in steroid secretion in a single run. By using a sample volume higher than those reported in other published multi-analyte ID-LC–MS/MS methods (15–20; 22), we were able to provide a general better sensitivity for the 9 hormones, determined not only as LLOQ, but also in a complex matrix, more similar to the real samples, as steroid free serum.

Such high sensitivity is very much needed for the careful definition of the lower reference limits, although DOC detection in the healthy population is still unsatisfying. However, in the routine application of our method, less sample volume may be used if the determination of steroids whose circulating levels are close to method sensitivity, like 11deoxycortisol, corticosterone, DOC, DHEA and progesterone, are not required. By increasing sample volume, noise and interferences may also be of relevance, and a second purification step on the perfusion column after SPE concentration was therefore adopted. The double purification guaranteed ruggedness across the analysis of many samples per run in several runs per week, helping to keep the system stable and clean and minimizing matrix interference. The chromatographic conditions guaranteed an adequate resolving power for isobaric compounds like corticosterone and 11deoxycortisol, DOC and 17OHprogesterone, respectively, and for the isotopic pattern cross-interferences between androstenedione and testosterone. The method also exhibited a good immunity from matrix components, as proved by European certified sera analyses and by the in-house validation.

The comparison of our ID-LC–MS/MS method with routine immunoassays employed in the clinical laboratory revealed a good agreement for ElecsysE170 in determining elevated levels of cortisol, testosterone in males and progesterone above 1 ng/ml. However, the ElecsysE170 performance was not reliable for low

levels of testosterone and progesterone both for lack of specificity and general overestimation, proving severely inadequate in depicting females' health status, especially for testosterone, thereby confirming other reports [5,8,28]. The huge overestimation obtained by Immulite2000 and DSL-9000 in measuring androstenedione and DHEA, respectively, is not only due to miscalibration between ID-LC–MS/MS and these immunoassays, but also to a sub-optimal specificity that may complicate evaluations in clinical borderline situations. In agreement with previous reports [16,29–31], we also demonstrated that the overestimation exhibited by 17OHP Bridge should be attributed to cross-reactivity, and finally to a severe lack of specificity in 17OHprogesterone measurement, being the ID-LC–MS/MS accuracy verified against reference material. Our present study took into account only one immunoassay per analyte; however we cannot exclude that other immunometric platforms or RIAs may perform better and give more consistent results.

Few steroid ID-LC–MS/MS reference intervals on adult populations have been published to date, and the topic remains a matter of debate. We therefore provided preliminary reference intervals for a wide pattern of steroids in an adult male and female population by analyzing 416 healthy drug-free normal-weight subjects. Our data showed a strong age-dependent decrease of androgens both in males and, to a greater extent, in pre-menopausal females. DHEA showed the highest rate of decrease in both sexes including post-menopausal females, whereas testosterone and androstenedione remained stable in post-menopausal women. The post-menopausal group showed an age-dependent increase in cortisol and 11deoxycortisol. In the male group, 17OHprogesterone and progesterone declined with age, but the sensitivity limit of progesterone probably masks an even higher extent of this tendency. In females, reference intervals were examined in the follicular phase, but more subjects distributed through the menstrual cycle are needed for the complete reference intervals of these hormones during physiological fluctuations.

Some discrepancies between our values and literature data could be due to sample collection: our subjects were saline infused for 10 min before blood withdrawal to avoid stress-related alterations in the glucocorticoid cascade. In particular, our cortisol and corticosterone intervals are lower than those reported by others, whereas 11deoxycortisol and 17OHprogesterone are generally higher [14,15,19,21]. Our data on 17OHprogesterone intervals in females cannot be compared with a previous work as no information on fertility was provided [15]. The intervals we obtained for androstenedione are in agreement with those reported by Kushnir in 2010 [22]. Male testosterone values are similar to those previously published [22,32–34], whereas the upper limit found in pre-menopausal females is generally lower than those reported [22,32–34]. Since circulating androgen levels are strongly age-related, comparison with cohorts of different ages is difficult. Multicenter studies involving a larger number of subjects grouped for decades are needed to establish age-related intervals and steroid trends. Not only the sampling procedures, but also ethnicity or statistical calculation of reference limits may account for differences among reports. A further confounding factor is the inclusion of subjects with BMI above 25. We took particular care to exclude overweight or obese subjects to define as normal values that are modified by subtle changes in body weight. However, the main cause of poor agreement among literature reports lies in the miscalibration and generally poor standardization of ID-LC–MS/MS methods, since reference procedures and matrix-based calibrators for most steroids are not available, and in-house validation alone is not sufficient to achieve a consensus [12,35]. To date, few studies have compared data from different ID-LC–MS/MS laboratories, and most focused on testosterone measurement [36,37]. Further comparative studies of steroid measurement are urgently needed to gain advances in biomedical research and enhance clinical care.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2010.11.005.

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