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# The role of sex steroid hormones in Crohn's disease

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Thesis presented to the Graduate course in Basic and Applied Immunology at the Ribeirão Preto Medical School - University of São Paulo, to obtain the degree of Doctor of Science - Concentration area: Basic and Applied Immunology.

Advisor: Prof. Dr. Cristina Ribeiro de Barros Cardoso

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*In loving memory of Ivonilda, my mother.*

*Em memória de Ivonilda, minha amada mãe.*

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***As blue as the sea***

*Ah, if the whole world could hear me*
*I've a lot to tell, say that I learned*
*And, in life, we have to understand*
*That one is born to suffer while the other laughs*

*But the one who suffers must always look for*
*At least find a reason to live*
*To see some meaning in life to dream*
*To have a dream all blue, as blue as the sea*

*(Tim Maia)*

***Azul da cor do mar***

*Ah, se o mundo inteiro me pudesse ouvir*
*Tenho muito pra contar, dizer que aprendi*
*E, na vida, a gente tem que entender*
*Que um nasce pra sofrer enquanto o outro ri*

*Mas quem sofre sempre tem que procurar*
*Pelo menos vir a achar razão para viver*
*Ver na vida algum motivo pra sonhar*
*Ter um sonho todo azul, azul da cor do mar*

*(Tim Maia)*

## ABSTRACT

**Background:** Crohn's disease (CD) is a chronic inflammatory disorder in the gastrointestinal tract of multifactorial etiology, being influenced by genetic predispositions, microbiota imbalance, and environmental factors. Considering that hormones are central regulators of human functions, the interaction between the endocrine and immune systems is of particular importance for homeostasis, with the capacity to modify several diseases course. Recently, several studies of sex steroid hormones immunomodulatory potential, such as dehydroepiandrosterone (DHEA), testosterone and estradiol, have been highlighted for a better understanding of immunoendocrine relationships and for clarifying the different outcomes of infectious or immune-mediated diseases, between male and female. **Aim:** To investigate the role of sex steroid hormones, androgens and estrogens, in Crohn's disease. **Results:** It was evaluated public transcriptome datasets (GSE100833 and GSE16879) of colonic biopsies from patients with CD, responders (RP) and non-responders (N-RP) to anti-TNF therapy, in addition to healthy controls (HC). Biopsies were collected before and after therapy. The analyzes were focused on endocrine pathways such as biosynthesis and metabolism of androgen and estrogen hormones, besides androgen (AR), estrogen alpha (ERα) and estrogen beta (ERβ) receptors signaling. The genes expression involved in the AR and ERα signaling pathways was predictive to the responsiveness to treatment, before the therapy, while other genes were able to differentiate only CD patients from HC. In the HC group, genes related to hormones inactivation and metabolism and also to the MAPK and ERBB pathways were more expressed, while others related to the hormones activation and formation were repressed. In patients, especially N-RP, pathways related to inflammation, such as IL-6, several chemokines and cell migration were induced. For *ex vivo* investigations, 39 CD and 20 HC individuals from HCFMRP/USP were recruited. Steroid hormones were measured by mass spectrometry in plasma samples. Cortisol, testosterone and DHEA were reduced in patients. With the aim of studying the mechanisms by which DHEA acts, peripheral blood mononuclear cells (PBMC) from eight HC were stimulated in vitro with anti-CD3 and anti-CD28 for three days, in the presence of DHEA and/or several antagonists to AR, ERα and ERβ. DHEA reduced the production of IL-6, IFN-γ and IL-10, but the specific blockade of ERβ restored the ability of PBMC to produce the cytokines. Finally, the potential of DHEA (40 mg/kg/day) to regulate intestinal inflammation *in vivo* was tested. For this purpose, experimental colitis was induced in male C57BL/6 mice with DSS 2.5% in drinking water by 10 days. Corroborating the other results, treatment with DHEA significantly attenuated the clinical score of the disease and other inflammatory markers, such as intestinal permeability, the total white blood cells count and the accumulation of neutrophils in the intestinal mucosa.
**Conclusion:** The non-responsiveness to anti-TNF in CD is related to sex steroid hormones and DHEA supplementation, probably via ERβ, has the potential to reduce the production of inflammatory cytokines, restoring the mucosal barrier and regulating intestinal inflammation.

**Silva, Murillo Duarte.** O papel de hormônios esteroides sexuais na doença de Crohn

## RESUMO

**Introdução:** A doença de Crohn (CD) é uma desordem inflamatória crônica no trato gastrintestinal de etiologia multifatorial, sendo influenciada por predisposições genéticas, desbalanço da microbiota, e fatores ambientais. Considerando que os hormônios são reguladores centrais das funções corporais, a interação entre os sistemas endócrino e imune é de especial importância para a homeostasia, com capacidade para modificar o curso de diversas doenças. Nos últimos anos, os estudos do potencial imunomodulador dos hormônios esteroides sexuais, como a dehidroepiandrosterona (DHEA), testosterona e estradiol têm se destacado tanto para o melhor entendimento das relações imunoendócrinas, quanto para esclarecimento dos diferentes desfechos de doenças infeciosas ou imuno-mediadas, entre o sexo masculino e feminino. **Objetivo:** Investigar o papel de hormônios esteroides sexuais, andrógenos e estrógenos na doença de Crohn. **Resultados:** Foram avaliados *datasets* de transcriptoma públicos (GSE100833 e GSE16879) de biópsias colônicas de pacientes com CD responsivos (RP) e não-responsivos (N-RP) à terapia com anti-TNF, além de controles saudáveis (HC). As biópsias foram coletadas antes e após a terapia. As análises foram focadas nas vias endócrinas como biossíntese e metabolismo de hormônios andrógenos e estrógenos, sinalização dos receptores de andrógeno (AR), estrógeno alfa (ERα) e estrógeno beta (ERβ). A expressão dos genes envolvidos nas vias de sinalização de AR e ERα foi preditiva em relação à responsividade ao tratamento, antes do mesmo ser implementado, enquanto outros genes foram capazes de diferenciar apenas os pacientes CD dos HC. No grupo HC, genes ligados à inativação e metabolização dos hormônios e às vias das MAPK e ERBB foram mais expressos, enquanto outros ligados à ativação e formação dos hormônios foram mais reprimidos. Nos pacientes, especialmente os N-RP, vias relacionadas à inflamação, como da IL-6, diversas quimiocinas e migração celular estavam induzidas. Para investigações *ex vivo*, foram recrutados 39 CD e 20 HC do HCFMRP/USP. Hormônios esteroides foram dosados por espectrometria de massa em amostras de plasma. Cortisol, testosterona e DHEA estavam reduzidos nos pacientes. Com o objetivo de estudar os mecanismos pelos quais o DHEA atua, células mononucleares de sangue periférico (PBMC) de oito HC foram estimuladas *in vitro* com anti-CD3 e anti-CD28 por três dias, na presença de suplementação com DHEA e/ou diversos antagonistas de AR, ERα e ERβ. O DHEA reduziu a produção de IL-6, IFN-γ e IL-10, mas o bloqueio específico do ERβ restaurou a capacidade das PBMC em produzir as citocinas. Finalmente, foi testado o potencial do DHEA (40 mg/kg/dia) em regular a inflamação intestinal *in vivo*. Para tal, foi induzida colite experimental em camundongos machos C57BL/6 com DSS 2,5% na água potável por 10 dias. Corroborando os demais resultados, o tratamento com DHEA atenuou significativamente o escore clínico da doença e outros marcadores inflamatórios, como a permeabilidade intestinal, a contagem de total de leucócitos circulantes e o acúmulo de neutrófilos na mucosa intestinal. **Conclusão:** A não responsividade ao anti-TNF na CD está relacionada aos hormônios esteroides sexuais e a suplementação com DHEA, provavelmente via ERβ, tem potencial em reduzir a produção de citocinas inflamatórias, restabelecendo a barreira e regular a inflamação intestinal.

# SUMMARY

# *1 Introduction*

# 1. INTRODUCTION

## *1.1 Crohn's disease*

Crohn's disease (CD) is part of the Inflammatory Bowel Diseases (IBD) together with Ulcerative Colitis (UC), in which disorders occur in the regulation of the mucosal immune response, favoring chronic inflammation in the gastrointestinal tract (GIT). Both diseases have a multifactorial etiology, being influenced by genetic predispositions, immunological tolerance failures, use of antibiotics, diet and lifestyle, among other factors.[1, 2]

CD is a very heterogeneous disease with a lager form of behavior. The intestinal lesions are characterized as transmural inflammation and can affect any part of the gastrointestinal tract, from the mouth until the anus. However, in mostly of the patients the disease is restricted to the ileum and/or colon.[2] Oral disease manifestation is more likely to be severe,[3] also is not uncommon perianal commitment, such as the development of fistulas, abscesses, fissures, skin tags, strictures, hemorrhoids and others.[4] Both are a signal to a poor disease prognosis, which impact the patient's quality of life and contribute significantly to increasing rates of morbidities in CD.[3, 5] The Montreal Classification for Crohn's disease summarize the disease manifestation (Table 1).[6]

The symptomologies are also diverse and depends on the behaviour and location that the disease will present. In periods of disease exacerbation, it is common signs and symptoms such as recurrent diarrhea, blood in the stool, low-grade fever, decreased appetite. Abdominal cramps can be of moderate to severe intensity.[7] Anemia can be observed due to loss of blood in the stool and therefore fatigue. In

addition, malabsorption of nutrients increases the fatigue manifestation, which can lead to weight loss and anorexia.[8, 9]

**Table 1 – Montreal Classification for Crohn's disease**

| | |
|---|---|
| ***Age at diagnosis:*** | |
| A1 | ≤ 16 years |
| A2 | 17-40 years |
| A3 | > 40 years |
| ***Disease location:*** | |
| L1 | Ileal |
| L2 | Colonic |
| L3 | Ileocolonic |
| L4* | Upper-isolated gastrointestinal |
| ***Disease behaviour:*** | |
| B1 | Non-stricturing and non-penetrating |
| B2 | Stricturing |
| B3 | Penetrating |
| p** | Perianal disease |

*L4 is a modifier that can be added concomitantly to L1-L3
**p is a modifier that can be added concomitantly to B1-B3

CD has a remarkable geographic distribution, in which the incidence and prevalence is higher in the "occidental world", North America and Europe. Thus, developed countries has the greater numbers of CD.[10] Indeed, the population's development is related to the CD epidemiology. The incidence increases according the development stage of the countries, also rural areas have lower prevalence than urban areas.[11] North America has the highest incidence of CD, 3.1-20.2 per 100,00, while Africa and South America has the lowest numbers, although increasing in the last decades.[10, 12] In Brazil, IBD notification still precarious and epidemiological data depend on scientific research, but not by a government mechanism. However, it is certain that currently the frequency of these diseases has been increasing in the Brazilian population.[13]

The CD development is an interplay among risk factors, such as genetic predisposition, microbiota dysbiosis and environmental factors.[1, 2] Among the genetic

risk factors, family history plays an important role to the CD development. In general, up to 10%-25% of the IBD patients have a first-degree relative previously diagnosed.[14] Other evidences of this aspect, is that Ashkenazi Jews have a three-to-four-times higher risk of CD diagnosis than non-Jews populations.[14] Also, monozygotic twins have a 20-50% CD establishment concordance rate, while for the dizygotic twins it is 10%.[15]

More than 200 genes alleles with single nucleotide polymorphism have been related to the IBD development risk factor.[16] Among them, the *NOD2* allele was the first described to increase the risk of CD and one of the most studied.[17, 18] European patients who carry one mutation (heterozygous) on *NOD2* have 2-3 fold increased risk of developing CD, while homozygous mutation increased the risk to 20-40 fold.[19] However, this risk is depending on the population, because *NOD2* variances has no significative role in East Asians populations.[16] Well, NOD2 protein is an intracellular pattern recognition receptor that sense bacterial components in the cytosol of the host to control invading microbiota, which is another relevant component to CD pathogenesis.[20]

The interactions between intestinal microbiota and components of immunity are essential for the chronic inflammation development.[21, 22] Under homeostasis conditions and tolerance in the GIT, the microbiota tends to induce regulation of the immune response and have lower potential to activate inflammatory mechanisms in the mucosa. However, the deregulation of the microbiota components, loss of species diversity, increase in pathogenic microorganisms, i.e., dysbiosis, is a fundamental part of the IBD pathogenesis.[21, 22] In fact, it is difficult to determine whether dysbiosis is

cause or consequence of the IBD development, but there is no doubt about its relevance and correlation with inflammatory molecular and cellular mechanisms triggered in IBD.[23]

It is well known that in CD the microbiota species diversity of the individual is diminished (α-diversity), along with decreased Bacteroides and Firmicutes bacteria.[21, 24] For example, *Faecalibacterium prausnitzii* (Firmicutes phylum) is reduced in CD patients, but it has a greater potential to regulate the mucosal inflammation by reducing the activation of nuclear factor kappa B (NF-κB) and consequently tumor necrosis factor alpha (TNF-α) production.[25, 26] On the other hand, invasive and high immune-activating bacteria are abundant in CD. One of the most common is the high prevalence of adherent-invasive *Escherichia coli* in CD patients,[27] which is specially associated to ileitis cases.[28]

Physical barriers are the first components of innate immunity that must be overcome by the microbiota for the inflammation initiation and/or worsening.[29] Normally, the mucus layer and intestinal epithelial cells prevent the contact of microorganisms from the intestinal lumen with the stroma, but dysbiosis favors the proliferation of mucolytic bacteria, consequently the mucus layer in inflammatory sites is thinner than that of non-inflamed tissues.[30, 31]

Bacterial translocation activates several cell types and pro-inflammatory factors are produced to combat invading pathogens.[32] Intestinal epithelial cells (IECs) in the paracellular sites are linked by tight junction proteins, such as occludins and claudins, which, when ruptured, expose molecular pattern receptors (PRRs). In the membrane of IECs PRRs are responsible for recognizing pathogen-associated molecular patterns

(PAMPs) that activate NF-κB and stimulate pro-inflammatory cytokines production, such as tumor necrosis factor (TNF).[32, 33] Moreover, augmented IECs death is a hallmark in IBD and along with increased TNF levels in the intestinal tissue. Indeed, TNF can induce epithelial cells apoptosis or necroptosis, which disrupt the intestinal barrier and promotes tissue injuries, that compromises the gut integrity and homeostasis.[34, 35, 36]

TNF is considered as a cytokine of the innate immune response and there are several other mechanisms that this cytokine can act to promote inflammation and, in consequence, disease worsening.[37] Firstly, besides EICs many are the source of TNF, such as macrophages, dendritic cells (DCs), T lymphocytes and fibroblasts. These own cells can be target to this cytokine, in addition to the endothelium. As consequence, TNF promotes angiogenesis and hypervascularization, activation of cells to produce more TNF (positive feedback-loop) and other inflammatory cytokines such as interleukin-1 (IL-1) and IL-6. Besides that, TNF promotes tissue destruction by inducing increased metalloproteinases production. Also, in T cells this cytokine induces resistance to apoptosis and thus accumulation of lymphocytes in the intestinal mucosa.[32, 38] Therefore, because TNF plays a central role in IBD pathogenesis, this cytokine is a target of one the most successful treatment to the disease.[39]

In this context, anti-TNF monoclonal antibodies are used in the DC and UC therapy in order to neutralize the biological effects of this cytokine.[39, 40] The main immunobiological used are Infliximab (IFX) and Adalimumab (ADA).[39] IFX is a chimeric IgG monoclonal antibody that has the Fc region and the constant chains of the Fab region of human origin, while the variable chains of the Fab region are murine.

ADA was the first fully human recombinant monoclonal antibody (IgGk1) approved for the treatment of inflammatory diseases.[41] Anti-TNF antibodies not only block soluble but also membrane cytokines, actually the neutralization of membrane TNF is the one fundamental to regulate the inflammatory processes in the intestinal mucosa.[42]

Anti-TNF agents are the most successful agents to induce corticosteroid free remission and maintenance of quiescent disease.[43] Also, choosing IFX as the first-line therapy to moderate-to-severe CD is superior to achieving clinical and endoscopic disease than conventional treatment, such as Azathioprine (AZA).[44] The monotherapy approach with AZA can be used for the maintenance of quiescent CD and in post-operative patients' recovery.[45] However, combination therapy with anti-TNF and AZA is recommended to a better disease prognosis, to improve the patients' overall condition and higher time of corticosteroid-free clinical remission than monotherapy with IFX or AZA.[46]

However, not all patients respond satisfactorily to anti-TNF treatment. It is known that genetic and environmental factors are involved in the predisposition to development of resistance. In this sense, about 20% of individuals with IBD are primary non-responders to this treatment strategy. In addition, around 30% of patients become resistant and no longer respond to drug administration over time.[39, 47]

Indeed, there are relevant differences in the immune response of non-responder patients compared to those who respond to anti-TNF administration and achieve clinical remission. Non-responsiveness is linked, for example, to the production oncostatin M in excess in the lamina propria, which acts in synergism with TNF inflammation chronification and tissue damage.[48] The lack of response to the

immunobiological agent may also be correlated with the composition of the microbiota intestinal tract, such as an increase in the Clostridiales order, which are related to a better prognosis for the disease.[49] Additionally, non-responders have decreased expression of antimicrobial genes, which help combat microbial translocation.[50, 51] Many polymorphisms in genes alleles are also correlated to primary non-response to anti-TNF in CD, showing that the non-responsiveness is not just an immune or environmental factor, but also genetic.[52]

The development secondary resistance is mainly due to the formation of antibodies against anti-TNF, which is an immunogenic protein.[53] It has been seen that the use of combination therapy with anti-TNF and another immunosuppressant, such as azathioprine, decreases the rate of secondary resistant patients. The probable reason why combined therapy favors the patient is that the use of another immunosuppressant concomitant with anti-TNF interferes with the production of antibodies against the immunobiological.[54]

Well, as described above, CD patients in combination therapy are more likely to have clinical remission and mucosal healing.[46] However, this combination is associated with relevant side effects, such an increased risk of lymphoma development and serious infections.[46, 55] Not only the long-term use of this drug may predispose to other complications such as liver cirrhosis,[56] hepatocellular carcinoma,[57] pancreatitis, polyarthritis or panniculitis syndrome (PPP),[58] but also the early administration of AZA may increase the adverse effects of this therapy, with no improvement on management.[59, 60] Furthermore, patients who use two concomitant

immunosuppressants have an increased risk of contracting serious infections and also developing lymphomas.[55, 61]

In addition, anti-TNFs are expensive drugs and greatly burden the Brazilian public health system, which provides these immunobiologicals to patients with IBD. Specifically in CD, it is projected that the Brazilian Government will spend an average of BRL 20,000 per patient, with accumulated expenses of BRL 82 million between 2013 and 2023. This amount counts only with the infusion of anti-TNFs, in addition to other outpatient costs and other procedures that the treatment of the disease demands.[62]

### *1.2 Sex steroid hormones and physiology*

Hormones regulates several essential processes in the human physiology, since fetal life through adulthood and old age. Disorders in the hormones production, distribution to the body and sensing are the etiology of a wide number of endocrine diseases. Furthermore, pathological processes of non-endocrine illness can alter hormone's normal function, worsening the disease course.[63, 64]

Among all the hormones there are a very characteristic family, the steroid hormones, formed by the steroidogenesis cascade.[65] The cholesterol molecule is the mold for the formation of these lipid hormones. Low-density lipoproteins (LDL) from the dietary are the major source of cholesterol to steroidogenesis cascade.[66] In this cascade are catabolized the progestagens, glucocorticoids and mineralocorticoids. From the progestagens are formed the sex steroids, such as the androgens and estrogens (Fig. 1).[65] The detailed steroidogenesis cascade for sex steroid hormones biosynthesis and metabolism is depicted on Fig. 2 to Fig. 4.

Well, whether the steroidogenesis began with the cholesterol and any of the steroid hormones can be formed from this molecule, what will determine which hormone will be produced is the enzymatic machinery that each tissue expresses. Thus, the expression of steroidogenic enzymes is a very regulated process to maintain the body homeostasis.[65] In addition, the number of chemical reactions to steroids production is much higher that the number of enzymes, that is because one single enzyme can perform a wide different reaction catabolizing and metabolizing several different hormones.[65, 67] Deficiencies in the production of this enzymes are the etiology of diseases, can be fatal and incompatible with life, making them essential to the physiology.[68]

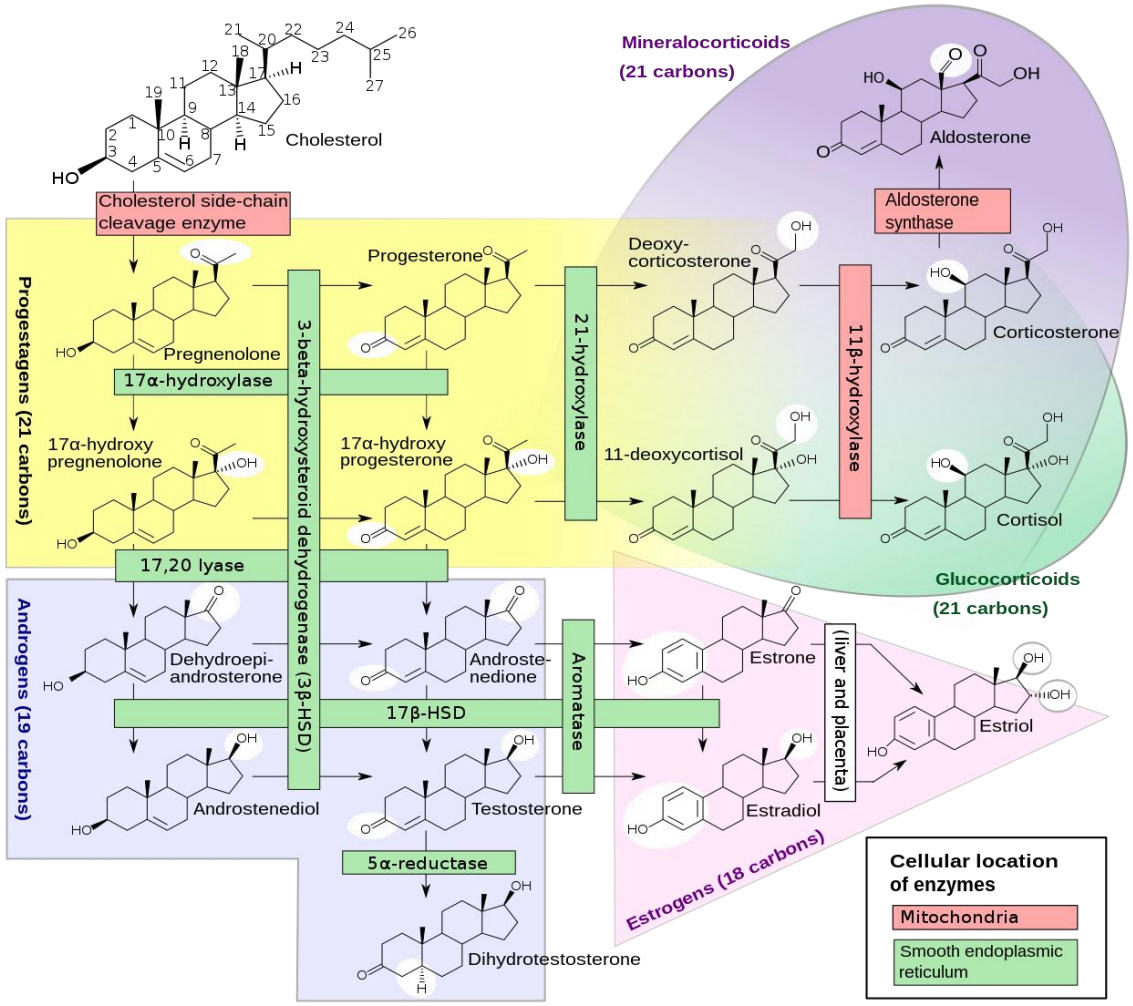



**Figure 1. Steroidogenesis cascade summary.** The steroidogenic acute regulatory protein (StAR) enzyme regulates the transfer of the cholesterol molecule from the cytoplasm to the mitochondria. In the inner mitochondrial space, the enzyme Cholesterol side-chain cleavage enzyme (P440scc) catalyzes the conversion of cholesterol to pregnenolone. In the endoplasmic reticulum, the enzyme 17 α-hydroxylase converts pregnenolone into 17α-hydroxy-pregnenolone, these being the progestagens of 21 carbons in its chains. All the glucocorticoids and mineralocorticoids also have 21 carbons. For the formation of the first androgens, the 17,20-lyase enzyme converts 17α-hydroxy-pregnenolone or 17α-hydroxy-progesterone into dehydroepiandrosterone (DHEA) or androstenedione, respectively. In this step, two carbons are lost, so all androgens are 19 carbons made. The enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) converts DHEA into androstenediol or androstenedione into testosterone. On the other hand, the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) catabolizes DHEA into androstenedione and androstenediol into testosterone. Finally, in peripheral tissues, testosterone can be transformed into dihydrotestosterone (DHT) by the enzyme 5α-reductase. For the formation of estrogen hormones, the enzyme aromatase is required, which converts the androgens androstenedione into estrone or testosterone into estradiol. The aromatase cuts out one carbon, thus all estrogens are made by 18 carbons. Furthermore, the hormone estrone can be converted into estradiol by the enzymatic activity of 17β-HSD. Finally, in the liver and, mainly, in the placenta, these estrogen hormones can be converted into estriol.[65]

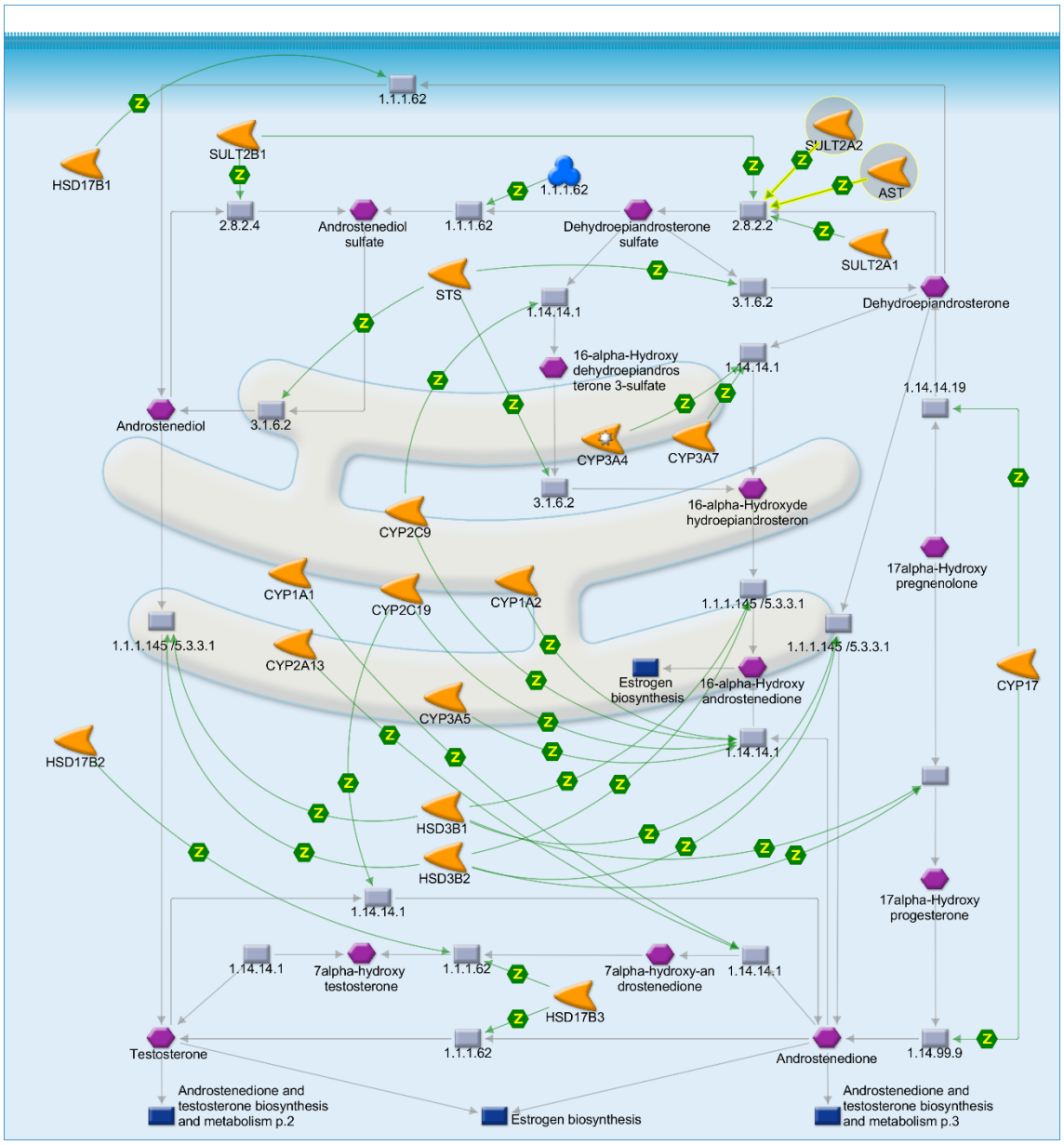



**Figure 2. Androgen hormones biosynthesis.** DHEA-S is converted in DHEA by the catalysis action of steroid sulfatase enzyme (STS; *STS*), while sulfotransferase 2A1 (ST2A1; *SULT2A1*) do the reverse way by conjugating sulfate to DHEA.[69] Further, DHEA can be reduced to androstenediol by 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1; *HSD17B1*).[70] The isoenzymes 3β-hydroxysteroid dehydrogenase 1/2 (3β-HSD; *HSD3B1* and *HSD3B2*, respectively) are responsible to convert DHEA into androstenedione and androstenediol into testosterone.[71, 72] Finally, androstenedione can be converted to testosterone by 17β-HSD3 enzyme (*HSD17B3*)[73] and 17β-HSD2 (*HSD17B2*) can make the opposite way by converting testosterone into androstenedione.[74] Figure obtained from the MetaCore software (Clarivate™).

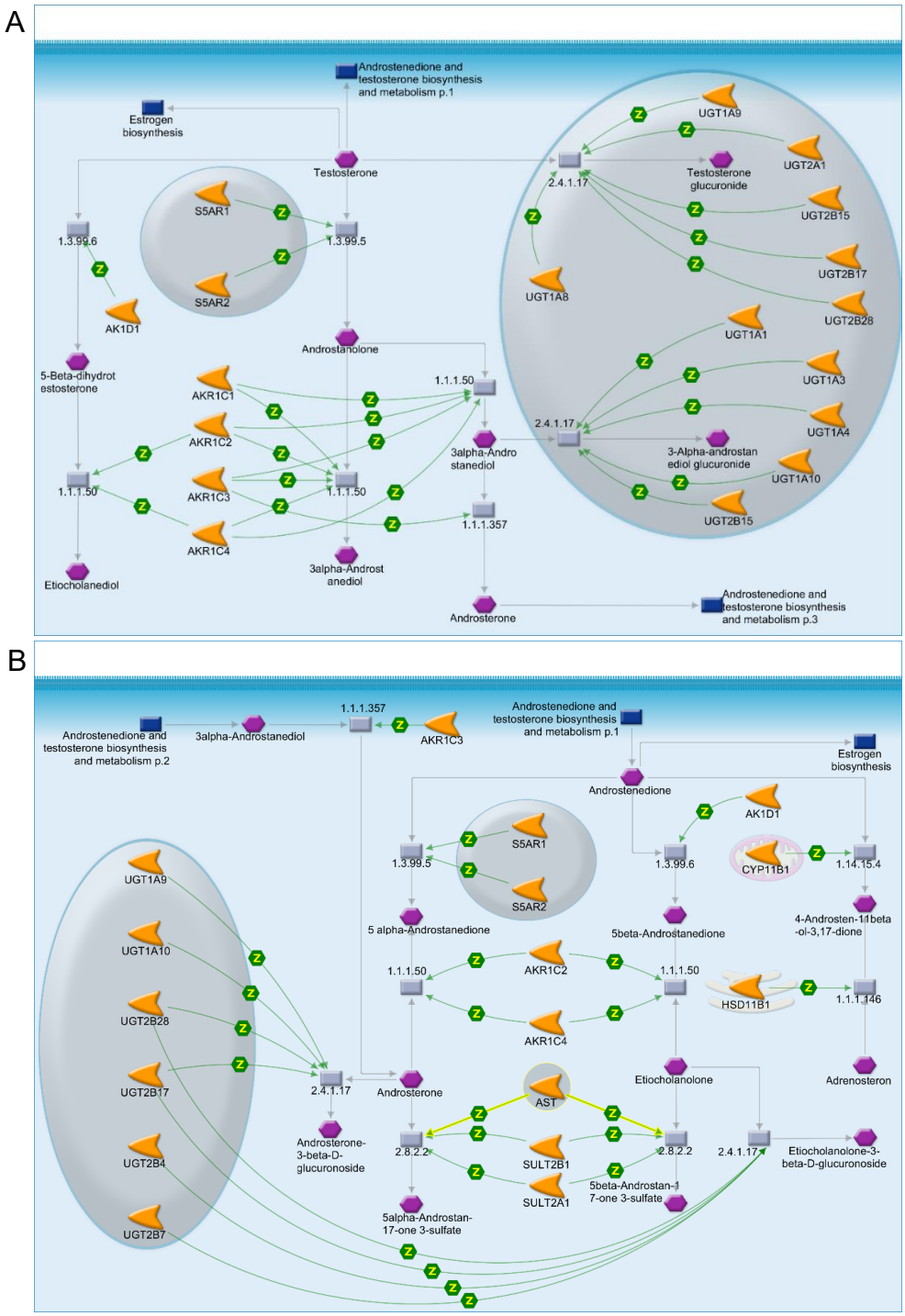
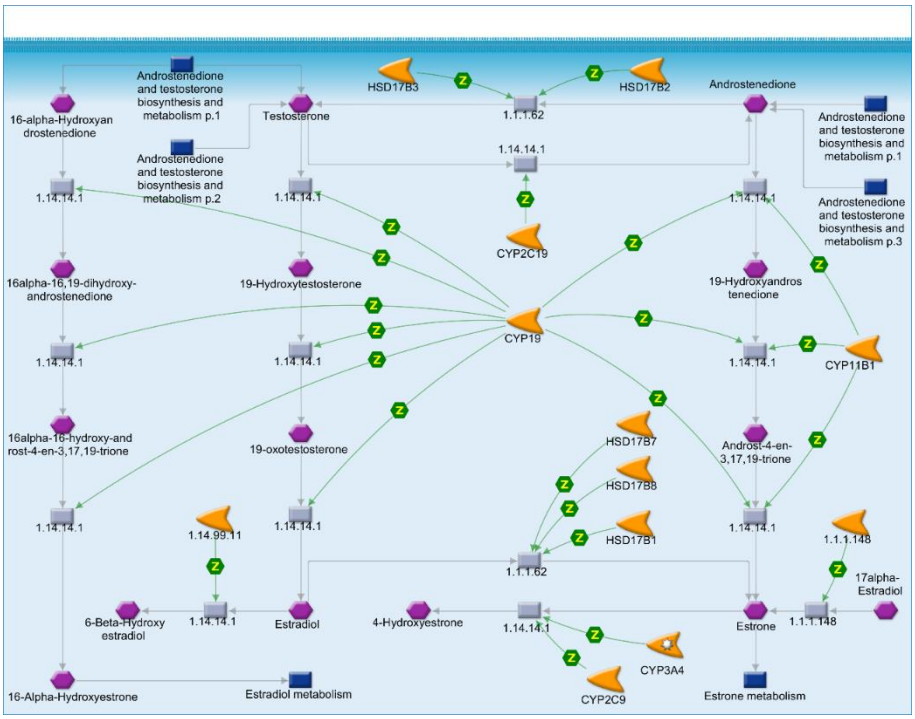



**Figure 3. Testosterone and androstenedione metabolism. (A)** Dihydrotestosterone (DHT), same as androstanolone, is formed from testosterone by the three isoenzymes steroid 5α-reductase 1,2,3 (*SRD5A1*, *SRD5A2* and *SRD5A3*, respectively).[75, 76] Also, DTH can be converted into a weaker androgen named androsterone by the enzyme aldo-keto reductase family member C3 (*AKR1C3*).[77] Finally, testosterone and 3α-androstenediol, a DHT metabolite, can be target to glucuronidation reaction. The UDP-glucuronosyltransferase (UGT) enzymes add a glucuronosyl group to the lipids hormones resulting in a more hydrophilic product, such as testosterone glucuronide and 3α-androstenediol glucuronide, facilitating the hormone excretion.[78] **(B)** Androstenedione can be converted into androsterone, which also is disponible to glucuronidation by the UGT enzymes facilitating the hormone metabolism.[79] Figure obtained from the MetaCore software (Clarivate™).

A

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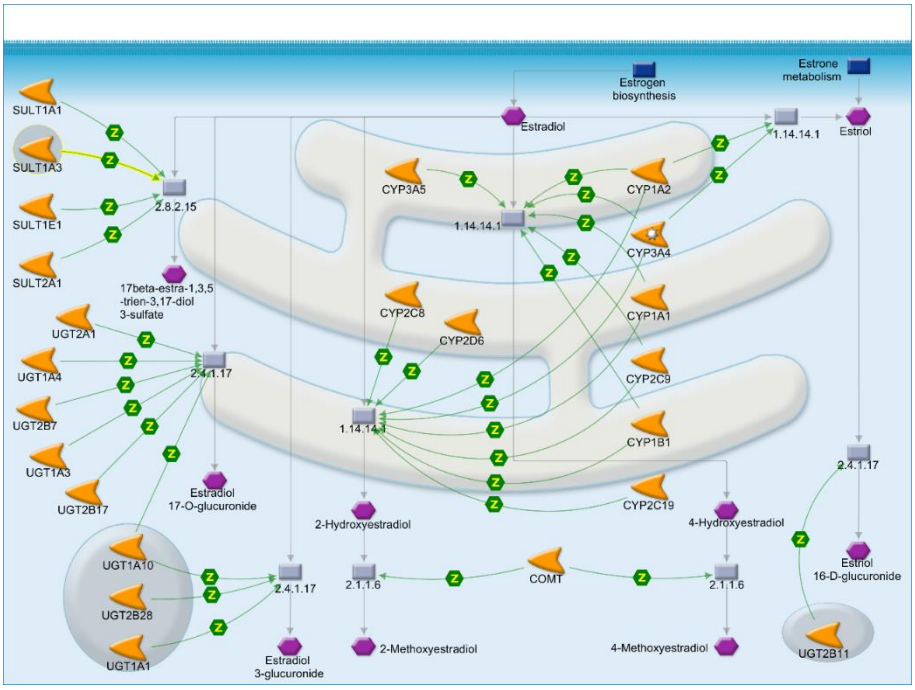


**Figure 4. Estrogen hormones biosynthesis and metabolism. (A)** The both androgens, testosterone and androstenedione, can be converted into estradiol and enthrone, respectively, by the aromatase enzyme (CYP19A1; *ARO*).[80] Also, the enzymes 17β-HSD1 and 17β-HSD7 (*HDS17B1* and *HDS17B7*, respectively) converts estrone into estradiol,[70, 81] while 17β-HSD7 (*HDS17B8*) converts estradiol into estrone.[82] **(B)** Estradiol can be converted into estriol and both hormones are target to the UGT enzymes to form estradiol 17-o-glucuronide and estriol 16-d-glucuroonide, thus facilitating the hormone metabolism.[79] Figure obtained from the MetaCore software (Clarivate™).

The androgens are known as "male hormones", whilst estrogens are "female hormones", due to its masculinization and femininization effect, respectively. Innate or late disorders on these hormones production can lead to development fetal issues, such as abnormalities of sex development and determination, hypogonadism or sterility.[83, 84] The hypothalamic-pituitary-gonadal axis (HPG) is responsible to induce and regulate the secretion of the major part of the circulating androgen and estrogen hormones, which is depicted in Fig. 5.

Testosterone is the main circulating androgen in men, approximately 95% of its production takes place in the testicles under luteinizing hormone (LH) stimulation, while the smallest part is peripherally produced (Fig. 5).[85, 86] However, just 1-2% of testosterone circulates in free form on the plasma, whilst the biggest amount circulates bounded to albumin or to sex hormone-binding globulin (SHBG).[85, 87] The "free-testosterone" and albumin-bounded can be referred as "bioavailable testosterone", because hormones bounded to SHBG usually do not dissociates to exert its biological action.[85, 88]

Moreover, among the androgen hormones, the dihydrotestosterone (DHT) is the stronger and with higher affinity to the androgen receptor (AR). Approximately, 20% of DHT can be formed in the testis, but the major part is formed in the peripherical tissues by the 5α-reductase enzymes that converts testosterone into DHT (Fig. 3A).[85, 89] It is not clear the relevance of circulating DHT, since just 0.8% is free and the half-life of this hormone is very low. Thus, the main relevance of DHT is on the peripheral tissues where it is formed.[90]

Likewise, estradiol is the most relevant estrogen in women and basically produced by the ovaries in until adulthood, as described in Fig. 5. Nonetheless, men also produce estrogen hormones, since Leydig cells also express aromatase enzyme (ARO), that converts testosterone into estradiol.[91] Thus, about 20% of estradiol is produced in the testes whereas 80% is formed in the peripheric tissues expressing ARO (Fig. 4A).[92, 93] Well, many physiological reproductive and non-reproductive functions in men are mediated by the transformation of testosterone into estradiol, which can bind the two isoforms of estrogen receptors, the alpha and beta (ERα and ERβ, respectively).[91, 94]

The hormone dehydroepiandrosterone (DHEA) is the first androgen produced in the steroidogenesis cascade and, despite having a low androgen effect, it is a relevant reserve for conversion into other sex hormones, through structural changes in the molecule (Fig. 1). Furthermore, DHEA is the most abundant steroid in both men and women. However, approximately 99% of serum DHEA is in the sulfated form (DHEA-S), which can be considered a DHEA reserve, since sulfate gives the molecule more stability and a longer serum half-life.[95, 96]

The primary source of DHEA is the adrenal cortex under hypothalamic-pituitary-adrenal axis (HPA) stimulation, depicted in Fig. 6. However, it is estimated that 10% to 25% of DHEA in men is formed in the testicles, while in women it is exclusively of HPA origin.[96] The relevance of adrenal DHEA production becomes more evident with age, in which the production of testosterone and estradiol is decreased in the gonads. Thus, the circulating DHEA is transported to the peripheric tissues and converted into testosterone and estradiol. Moreover, 100% of the estradiol

in post-menopausal women is from DHEA metabolism, whilst approximately 50% of the circulating androgens in men adulthood is also produced by the HPA axis.[97]

Regarding the sex steroid hormones receptors, the most explored are AR, ERα and ERβ, which are cytosolic receptors and mediate genomic responses, i.e., slow responses, better described in Fig. 7. Besides the role on embryonic sex development and masculinization/femininization phenotypes outcome, these receptors are well known to influence the cell cycling and differentiation. Part of these functions are regulated by the genomic effects of the sex steroid receptors, but AR/ERs also plays non-genomic functions of rapid response.[98, 99] However, the non-genomic alterations mediated by androgen and estrogens hormones and their receptors is not the focus of this study.

Well, the role of AR appears to be dependent of the tissue and stage of cell development. The absence of AR can impair immature cells differentiation and induce over proliferation, thus increasing cancer cell growth, which ensures that AR is required for normal function.[100, 101] In contrasting, the expression of AR in cancer cells supports cell survival and proliferation.[102, 103] It worth to say that most of these studies were conducted on prostate cancer cells. The actions of the ERs are easier to track, since ERα and ERβ has antagonic functions, in which ERα enhances cell proliferation and resistance to cell apoptosis, whereas and ERβ regulates the ERα expressions, abrogate cell proliferation and activate apoptotic cascades.[104]

In view of this, drugs that blocks AR/ERs signaling pathways are used as chemotherapies in attempt to abrogate cancer cell proliferation.[105, 106] Flutamide is an example of drug that antagonize AR; Finasteride blocks the 5α-reductase enzyme,

dampening the stronger androgen formation DHT;[105] Fulvestrant is widely used as full antagonist of ERα and ERβ.[106] Thus, these drugs can be used to treat some kind of cancers and, of course, these therapy strategies are just elective to tumors that express these receptors.

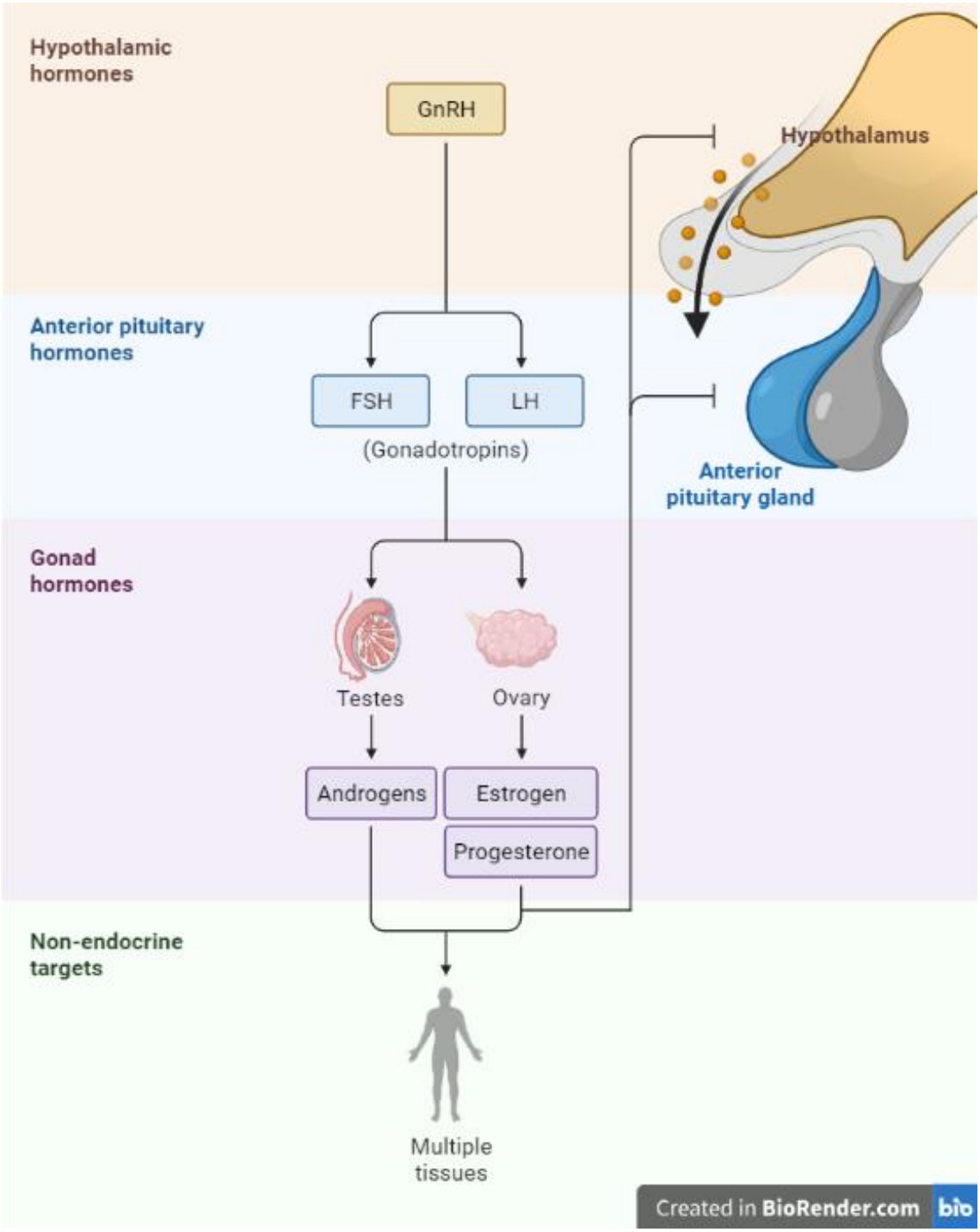


**Figure 5. Hypothalamus-pituitary-gonadal axis (HPG).** Gonadotropin-releasing hormone (GnRH) are produced by neurons within the hypothalamus and released to stimulate the production of gonadotropins in the anterior pituitary gland, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In males, FSH binding its receptor expressed on Sertoli cells to induce the spermatogenesis, whilst LH has as target the Leydig cells. The main function of LH in the testes is to induce the expression of enzymes necessary for the biosynthesis of testosterone from the cholesterol molecule. In females, the LH increases the expression of the StAR and 3β-HSD-II in Theca cells to androstenedione production. Thus, androstenedione is released and internalized in Granulosa cells. Well, FSH has as function to induce the expression of aromatase in the Granulosa, which will convert androstenedione into estradiol. Finally, the produced hormones testosterone and estradiol regulates the release of GnRH and gonadotropins in a negative feedback loop.[107, 108]

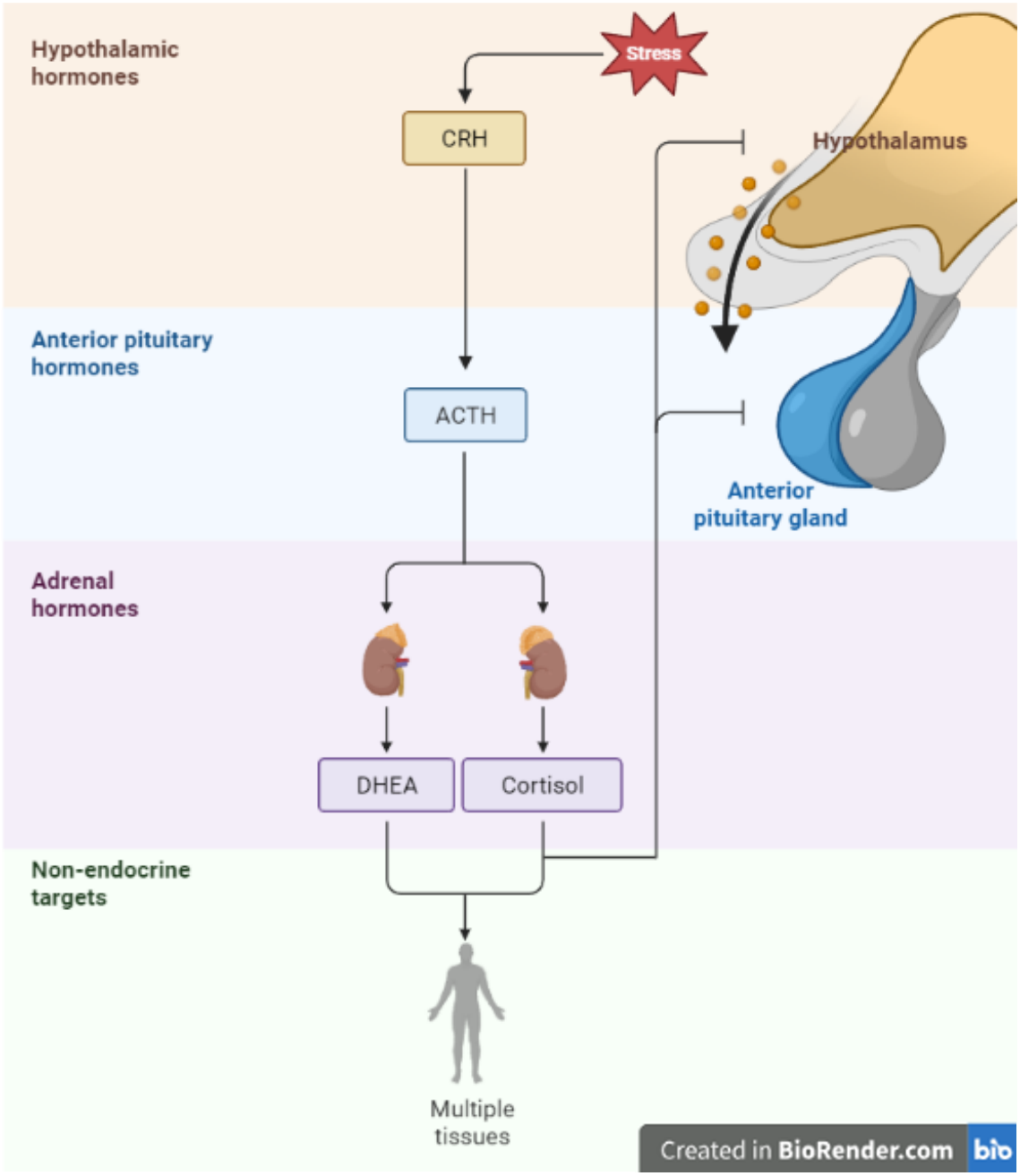


**Figure 6. Hypothalamus-pituitary-adrenal axis (HPA).** The circadian cycle and stressor factors, not just behavioral, but also inflammatory such as IL-6 and TNF, induces the production of corticotropin releasing hormone (CRH) in the hypothalamus. CRH reaches the anterior pituitary gland to induces the secretion of adrenocorticotropic hormone (ACTH). In the adrenal cortex the ACTH increases the cholesterol capture in the mitochondria to pregnenolone formation, which can be converted into cortisol or dehydroepiandrosterone (DHEA). Finally, the produced hormones cortisol and DHEA regulates the release of CRH and ACTH in a negative feedback loop.[109]

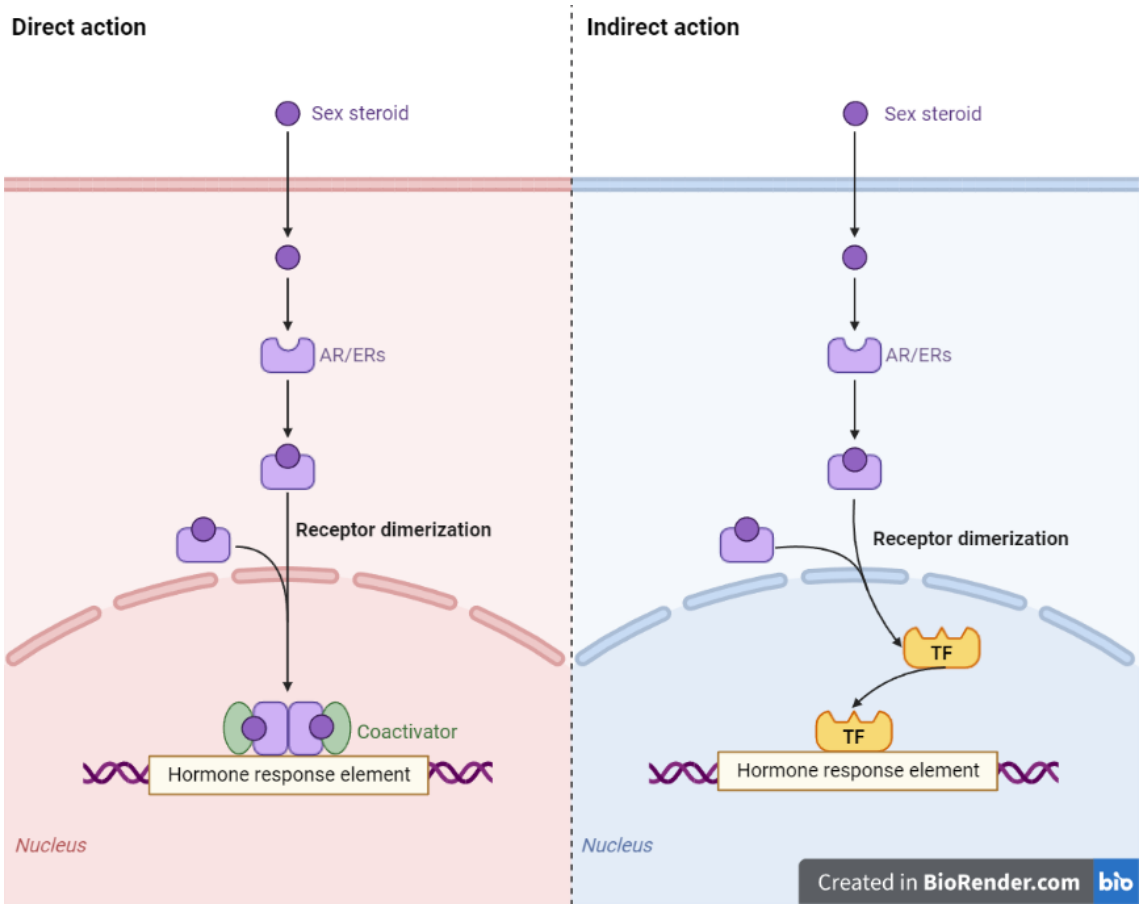


**Figure 7. Genomic action of sex steroid hormones.** The lipid-soluble hormones diffuse across the cell membrane and binds its receptors (AR or ERs). Chaperone proteins maintain the receptors in homodimers forms in absence of their related ligands, but after hormone binding the chaperone is dissociated and the receptor translocate to the nucleus. Each sex steroid receptor has at least three domains; a binding domain to hormone ligand; a DNA-binding domain; a transactivation domain. In the direct action, the AR/ERs binds specific androgen/estrogen responsive element (AREs and EREs, respectively) and recruits several coactivators proteins, that mediate epigenetic changes on the DNA for activate or repress gene expression, i.e., the AR/ERs acts as a transcription factor. On the other hand, the complex of AR/ERs can activate second messengers, transcription factors, that will mediate the gene expression on indirect action of the sex steroid hormone receptors.[110, 111]

# 2 *Hypothesis*

## 2. HYPOTHESIS

In Crohn's disease there are immunoendocrine biomarkers that influence the disease course, which involve the sex steroid hormones, such as dehydroepiandrosterone, testosterone and estradiol, and the signaling pathways of androgen and estrogen receptors.

# 3 Objectives

## 3. OBJECTIVES

### *3.1 Main objective*

To investigate the modulation of various aspects of the immune response in Crohn's disease, especially the non-responsiveness to the treatment with anti-TNFs, by sex steroid hormones (dehydroepiandrosterone, testosterone and estradiol) and their receptors (AR, ERα and ERβ).

### *3.2 Specific objectives*

3.2.1 To investigate the role of the "sex steroid axes" in the non-responsiveness to anti-TNF therapy;

3.2.2 To evaluate the sex steroid hormone imbalance in Crohn's disease patients;

3.2.3 To determine a sex steroid hormone as target to further detailed investigations;

3.2.4 To study the immunoregulator potential of DHEA in *in vitro* assays;

3.2.5 To investigate a pathway which DHEA could be acting;

3.2.6 To analyze the potential of DHEA to treat *in vivo* experimental colitis.

# 4 Materials and Methods

## 4. MATERIALS and METHODS

### *4.1 Bioinformatic*

The datasets GSE100833[112] and GSE16879[51] were collected from the Gene Expression Omnibus (GEO) and analyzed on GEO2R.[113, 114] The GEO2R applies forced normalization along with limma package v3.26.8.1.[112] to data analysis. Benjamini & Hochberg (false discovery rate) was used to calculate the p-values. GEO2R uses version 3.2.3 of R to perform the analysis. Differentially expressed genes (DEGs) were obtained from GEO2R for both datasets. The analysis of the GSE100833 were conducted in GEO2R by generating Volcano plots showing the DEGs and Uniform Manifold Approximation and Projection (UMAP) graphs.

The array intensity matrix and metadata were accessed from GEO using getGEO function of GEOquery package (version 2.62.2). The limma package (version 3.50.3) was used for normalization and differential expression analysis. The collapseRows function of WGCNA package (version 1.71) was employed for collapse of probes ids to respective genes using MaxMean method. After pre-processing, normalization and exclusion of genes with null variance in intensities we obtained a total of 22188 genes. Analyzes were performed in R (version 4.1.2) in the Rstudio (version 2021.09.2 build 382). The "endocrine axes" were obtained from the MetaCore software (Clarivate™) with the gene list (Table 2) and their respective figures (Fig. 2-4 and Fig. 9-11).

**Table 2 – Endocrine axes gene list obtained from MetaCore software (Clarivate™).**

| Androgens biosynthesis and metabolism | AR signaling pathway | | Estrogens biosynthesis and metabolism | ESR1 signaling pathway | | ESR2 signaling pathway |
|---|---|---|---|---|---|---|
| *AKR1C1* | *ACP3* | *KLK2* | *COMT* | *AKT1* | *MAP2K4* | *AKT1* |
| *AKR1C2* | *ADAM10* | *KLK3* | *CYP11B1* | *AKT2* | *MAP2K7* | *AKT2* |
| *AKR1C3* | *ADAM17* | *KLK4* | *CYP19A1* | *AKT3* | *MAP3K5* | *AKT3* |
| *AKR1C4* | *ADAM9* | *KRAS* | *CYP1A1* | *BIRC5* | *MAPK1* | *ARNT* |
| *AKR1D1* | *AKT1* | *MAP2K1* | *CYP1A2* | *CASP4* | *MAPK10* | *BTG2* |
| *CYP11B1* | *AKT2* | *MAP2K2* | *CYP1B1* | *CCNA2* | *MAPK8* | *CCNA2* |
| *CYP17A1* | *AKT3* | *MAPK1* | *CYP2C19* | *CCNE1* | *MAPK9* | *CCNB1* |
| *CYP1A1* | *APP* | *MAPK3* | *CYP2C8* | *CD44* | *MMP9* | *CCND1* |
| *CYP1A2* | *AR* | *MDM2* | *CYP2C9* | *CDKN1A* | *MYC* | *CCNE1* |
| *CYP2A13* | *BAK1* | *MIR125B1* | *CYP2D6* | *CHUK* | *NCOA3* | *CDC25A* |
| *CYP2C19* | *BCL2L1* | *MIR125B2* | *CYP3A4* | *CXCL12* | *NFKB1* | *CDKN1A* |
| *CYP2C9* | *BRAF* | *MMP7* | *CYP3A5* | *EGFR* | *NFKBIA* | *CDKN1B* |
| *CYP3A4* | *CASP2* | *MYC* | *HSD17B1* | *ELK1* | *NFKBIB* | *ERBB2* |
| *CYP3A5* | *CAV1* | *NCOA1* | *HSD17B2* | *ESR1* | *NFKBIE* | *ERBB3* |
| *CYP3A7* | *CBL* | *NCOA2* | *HSD17B3* | *ESR2* | *PIK3CA* | *ESR1* |
| *CYP3A7-CYP3A51P* | *CCND1* | *NCOA3* | *HSD17B7* | *FBLN1* | *PIK3CB* | *ESR2* |
| *HSD11B1* | *CDH1* | *NR3C1* | *HSD17B8* | *FN1* | *PIK3CD* | *FOXM1* |
| *HSD17B1* | *CDH2* | *NRAS* | *SLX1A-SULT1A3* | *FOS* | *PIK3R1* | *GADD45A* |
| *HSD17B2* | *CDKN1A* | *PDPK1* | *STS* | *FOSL1* | *PIK3R2* | *HIF1A* |
| *HSD17B3* | *CFLAR* | *PELP1* | *SULT1A1* | *GNRH1* | *PIK3R3* | *ID1* |
| *HSD3B1* | *CLU* | *PIK3CB* | *SULT1A3* | *GNRHR* | *PLAU* | *ITGA1* |
| *HSD3B2* | *CXCL8* | *PIK3R2* | *SULT1A4* | *GPER1* | *RELA* | *JUN* |
| *SRD5A1* | *EGF* | *PRKCD* | *SULT1E1* | *IGFBP3* | *SRC* | *MYC* |
| *SRD5A2* | *EGFR* | *PSAP* | *SULT2A1* | *IKBKB* | *STAT3* | *NCOA2* |
| *SRD5A3* | *ELK4* | *PSCA* | *SULT2B1* | *IQGAP1* | *TERC* | *NCOR1* |
| *STS* | *ERBB2* | *PTEN* | *UGT1A1* | *KRT4* | *TERT* | *PDGFB* |
| *SULT2A1* | *ESR2* | *PTPN11* | *UGT1A10* | *KRT7* | *TFF1* | *PTEN* |
| *SULT2B1* | *ETV1* | *RAF1* | *UGT1A3* | *LEP* | *TGFBI* | *RBL2* |
| *UGT1A1* | *EZR* | *RB1* | *UGT1A4* | *LEPR* | *TRAP1* | *SKP2* |
| *UGT1A10* | *F2R* | *RLN1* | *UGT2A1* | *MALL* | *YWHAQ* | *SP1* |
| *UGT1A3* | *FEN1* | *SCAP* | *UGT2A2* | | | *VEGFA* |
| *UGT1A4* | *FGF1* | *SGK1* | *UGT2B11* | | | |
| *UGT1A8* | *FGF2* | *SHC1* | *UGT2B17* | | | |
| *UGT1A9* | *FGF8* | *SKP2* | *UGT2B28* | | | |
| *UGT2A1* | *FGFR1* | *SLC45A3* | *UGT2B7* | | | |
| *UGT2A2* | *FGFR2* | *SOS1* | | | | |
| *UGT2B15* | *FOLH1* | *SOS2* | | | | |
| *UGT2B17* | *FOXA1* | *SPRY1* | | | | |
| *UGT2B28* | *FRS2* | *SPRY2* | | | | |
| *UGT2B4* | *FRS3* | *SRC* | | | | |
| *UGT2B7* | *GAB1* | *SRD5A1* | | | | |
| | *GRB2* | *SRD5A2* | | | | |
| | *HRAS* | *STAT3* | | | | |
| | *IGF1* | *STEAP1* | | | | |
| | *IGF1R* | *TERT* | | | | |
| | *IGFBP3* | *TGFB1* | | | | |
| | *IL6* | *TLR2* | | | | |
| | *IL6R* | *TMPRSS2* | | | | |
| | *IL6ST* | *TP53* | | | | |
| | *IRS1* | *VCAN* | | | | |
| | *JAK1* | *VEGFA* | | | | |
| | *KLF5* | | | | | |

### *4.2 Crohn's disease patient's and healthy controls cohort*

Thirty-nine (39) CD patients were recruited from the Inflammatory Bowel Disease's Outpatients Care of the Clinics Hospital from the Ribeirão Preto Medical School of the University of São Paulo, Brazil. The healthy control (HC) subjects (n =

20) were recruited from the same institution. All participants were male. The inclusion criteria for the patients were use of anti-TNF (IFX) or IFX plus AZA in combination therapy. The exclusion criteria for CD and HC individuals were determined as not presenting active infections, autoimmune, hematological, neoplastic or endocrine diseases. We also excluded those who used or have been using glucocorticoids in the last three months and those treated with three or more immunosuppressant drugs. The Harvey-Bradshaw Index (HBI) and Simple Endoscopic Score for Crohn's Disease (SES-CD) were used to clinical and endoscopic evaluation, respectively.[115]

### *4.3 Blood collection and PBMC isolation*

Peripheral venous blood (20 mL, BD Vacutainer®, SodiumHeparinN, Franklin Lakes, NJ, EUA) were collected from all participants for the immunological assays. The blood was centrifuged (4°C, 220 g, 5 min) and plasma samples were stored at -80ºC until analysis. The centrifuged blood was diluted twice in incomplete RPMI 1640 medium (Gibco BRL, Grand Island, USA), supplemented with 23.8µM sodium bicarbonate (Sigma-Aldrich, Saint-Louis, MO, USA) and 10.9µM HEPES (Sigma - Aldrich, Saint-Louis, MO, USA), followed by the addition of Ficoll-PaqueTM PLUS (GE Healthcare, Sweden) for isolation of peripheral blood mononuclear cells (PBMC). For that, centrifugation was performed for 30 min, at 25°C, 220 g. The cells were carefully collected, washed with incomplete RPMI 1640, then suspended in 10 mL of complete RPMI 1640 medium enriched with 10% heat inactivated fetal bovine serum (SBF) (Gibco BRL, Grand Island, USA), 200 µM L-glutamine (Sigma-Aldrich, Steinheim, Germany), 10,000 U/mL penicillin (Gibco – Invitrogen, Ontario, Canada), 10,000 µg/mL streptomycin (Gibco - Invitrogen, Ontario, Canada), 2-mercaptoethanol

50 µM (Sigma-Aldrich, Saint-Louis, MO, USA), 100 µM sodium pyruvate (Sigma-Aldrich, Saint-Louis, MO, USA), non-essential amino acids 10 mL/L (Sigma-Aldrich, Saint- Louis, MO, USA). The mononuclear cells were counted and evaluated for viability by 0.2% Trypan Blue staining in a Neubauer chamber, followed by culture experiments.

### *4.4 DHEA in vitro metabolization*

To test the *in vitro* metabolization of DHEA 5.0 µM (Sigma-Aldrich, Saint-Louis, MO, USA), PBMC ($5x10^5$ cells/well) from CD and HC described above were cultured in complete RPMI medium, in triplicate, 96-well culture plates, at 37°C, 5% CO2 for 5 days and stimulated with anti-CD3 and anti-CD28 (1.0 µg/mL). After this period, the culture supernatant was stored at -80°C until hormones measurement.

### *4.5 Steroid hormones measurement by Mass Spectrometry (LC-MS/MS)*

The mass spectrometry methodology used here quantified the total plasma and supernatant steroid hormones and comprised dissociation steps for proteins removal, to increase the measurement precision of the hormones.

Chromatographic separation was performed on a Nexera High Performance Liquid Chromatographic (HPLC) system by Shimadzu (Kyoto, HO, JP) according to Peti et. al, 2018.[116] Mobile phases used were (A) formic acid 0.1% in Water and (B) formic acid 0.1% in acetonitrile. It was used the Ascentis Express C18 column (100 x 2.1 mm; 2.7 µm) from Supelco (St. Louis, MO, USA), along with a 10 µL injection volume and a 0.5 mL/min flow rate. The gradient condition was 1 min. of 30% phase B, followed by an increase to 50% for 2 min, and then 60% for 5 min, which was then

maintained until 8 min. Afterwards, it was increased to 98% phase B (9 min), thus maintaining this condition until 11 min. The MRM channel-like settings, the collision energy and declustering potential were optimized for individual's hormone targets.

The mass spectrometry analysis was proceeded using a TripleTOF® 5600+ (Sciex -Foster, CA, USA), in positive mode operation. The established parameters were gas1: 60 psi; gas2: 40 psi; curtain gas: 25 psi; ion spray voltage: 5 kV; turbo temperature: 550 $^{o}$C; range of m/z for product ions experiments: 50 a 400; and dwell time: 100 ms. The atmospheric-pressure chemical ionization (APCI) Negative Calibration Solution (Sciex – Foster, CA, USA) was used to mass calibration (< 2 ppm). Data was acquired with AnalystTM Software (SCIEX- Foster, CA, USA). The hormones' identification was performed with PeakViewTM (SCIEX- Foster, CA, USA) and their quantification was made by MultiQuantTM software (SCIEX-Foster, CA, USA), which normalizes the peak intensities of individual molecular ions according to an internal standard for each steroid hormone. The lipid hormones' final concentration was normalized by the initial volume of plasma and the results were expressed as nanograms per deciliter (ng/dL).

### *4.6 PBMC culture assay*

Eight HC donors were selected to test the *in vitro* DHEA 5.0 µM mechanism of action. For that PBMC (5x10$^{5}$ cells/well) were cultured in complete RPMI medium, in triplicate, 96-well culture plates, at 37°C, 5% CO2 for 3 days and stimulated with anti-CD3 and anti-CD28 (1.0 µg/mL). Flutamide 10 µM, Fulvestrant 100 nM, MPP dihydrochloride 100 nM and PHTPP 150 nM (Sigma-Aldrich, Saint-Louis, MO, USA) were used in combinations with DHEA. (Fig. 24) After this period, the culture

supernatant was stored at -80ºC until cytokines measurement. The PBMC were kept in TRIzol™ to RNA extraction, the qPCR will be further performed.

### *4.7 CACO-2 culture assay*

CACO-2 cells were selected to test the *in vitro* DHEA 5.0 µM mechanism of action in an epithelial intestinal cell. For that CACO-2 ($2x10^5$ cells/well) were cultured in complete RPMI medium, in quadruplicate, 24-well culture plates, at 37°C, 5% CO2 for 24 hours and stimulated with lipopolysaccharide (100 ng/mL). Flutamide 10 µM, Fulvestrant 100 nM, MPP dihydrochloride 100 nM and PHTPP 150 nM (Sigma-Aldrich, Saint-Louis, MO, USA) were used in combinations with DHEA. (Fig. 24) After this period, the culture supernatant was stored at -80ºC until cytokines and hormones measurement. The CACO-2 cells were kept in TRIzol™ to RNA extraction, the qPCR will be further performed. In addition, after the culture, CACO-2 cell were lysed to protein extraction and BD™Phosflow performing.

### *4.8 CACO-2 proliferation/viability assay*

CACO-2 cells proliferation/viability was quantified by ATP measurement. For that CACO-2 ($2x10^4$ cells/well) were cultured in complete RPMI medium, in octuplicate, 96-well culture plates, at 37°C, 5% CO2 for 48 hours and stimulated with lipopolysaccharide (100 ng/mL). DHEA 5.0 µM, Flutamide 10 µM, Fulvestrant 100 nM, MPP dihydrochloride 100 nM and PHTPP 150 nM (Sigma-Aldrich, Saint-Louis, MO, USA) were used in combinations with DHEA. (Fig. 24) After this period, the cells were lysed and the luminescence read in a luminometer according the instructions of the CellTiter-Glo® 2.0 Assay (Promega, Madison, WI, USA).

### *4.9 Dosage of cytokines*

The cytokines interleukin-6 (IL-6), IL-10, IFN-γ and TNF were quantified in supernatant cultures or mice intestinal tissue. ELISA Set (BD Biosciences, San Jose, CA, USA) were used following the manufacturer's specifications.

### *4.10 Myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) assay*

For neutrophils quantification, the activity of the MPO enzyme in the intestinal tissues was evaluated. Intestine segments were cut into small pieces, weighed and immersed in a buffer solution containing sodium phosphate (0.02M) and EDTA (5.58g/L) for homogenization. Then, the tissue was centrifuged at 600xg for 15 minutes at 4°C and the supernatant discarded. The pellet was resuspended by vortexing in a buffer solution containing sodium phosphate and EDTA, and ice-cold 0.2% NaCl solution was added to lyse the red blood cells. The pellet obtained after centrifugation was resuspended in a buffer solution containing sodium phosphate and 0.5% H-TAB detergent (Hexadecyl-Trimethyl Ammonium Bromide, Sigma-Aldrich, USA). To finalize enzyme extraction, the sample was frozen and thawed three times and centrifuged at 12000xg for 15 minutes at 4°C. The supernatant was used for the enzymatic assay performed in 96-well plates containing 50µL of the sample, which was revealed by adding TMB (3.3',5.5'-tetramethylbenzidine, BD PharmigenTM, San Diego, USA) and incubated at 37°C for 15 minutes in the dark. The reaction was interrupted by the addition of 4M H2SO4, and the reading was performed in a spectrophotometer at 450nm.[117]

The same supernatant used for MPO analysis was used to macrophage quantification by NAG assay. For this, 25µL of the supernatants placed in 96-well

plates were used, 25µL of 2.24mM NAG solution (p-nitrophenyl-2-acetamide-β-D-glucopyranoside) and 100µL of citrate buffer (50 mM, pH 4.5). Next, the plate was incubated at 37°C for 60 minutes. Finally, the reaction was interrupted by the addition of 100µL of glycine buffer (200 mM, pH 10.4), and the reading was performed in a spectrophotometer at 405nm.[118]

### *4.11 DHEA preparation*

The components used to prepare the nanoemulsion were: poloxamer 188 (Sigma-Aldrich, St Louis, MO, USA), medium chain triglyceride (MCT), N-1, 2-dioleoyloxy-3-trimethylammonium-propane chloride (DOTAP) and egg phospholipids with 80% phosphatidylcholine (Lipoid E80 – Ludwigshafen, Germany), polysorbate 80 and the hormone dehydroepiandrosterone (Sigma-Aldrich, St Louis, MO, USA). The water used in all preparations was Milli-Q type with a conductivity of 0.055 S/cm (Millipore, France).[119]

The nanoemulsion with DHEA was composed of two phases: oily and aqueous. The oily phase was composed of 15% of MCT, 1% of lipoid E80, 0.2% of DOTAP and the drug DHEA at a concentration of 8.8 mg/mL, while the aqueous phase 5% of polysorbate 80 and aqueous solution of poloxamer 0.1% in sufficient quantity to prepare 20 mL of formulation. In preparing the nanoemulsion, the aqueous phase was poured over the oil phase, both at 70°C, under mechanical agitation (IKAWorks Inc., Wilmington, NC, USA) for 5 min. Then, it was subjected to low-frequency ultrasound (20 kHz, VCX 500; Sonics Materials Inc., Newtown, CT, USA) for 5 min with an amplitude of 50% and pulsed, with 1 s on and 1 s off. The white nanoemulsion was also prepared in the same way, but without the addition of the DHEA.[119]

### *4.12 Experimental colitis*

Male C57BL/6 mice, between 7 and 8 weeks old, from the Central Animal Facility – University of São Paulo, Ribeirão Preto, were used. The mice were transferred and placed in the vivarium of the Laboratory of Immunoendocrinology and Regulation (LIR) of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, in microisolators with water and food sterilized *ad libitum* throughout the experiment. The experimental colitis was induced with dextran sulfate sodium (DSS – Biomedicals, Illkirch, France) given ad libitum in water to the mice by 10 days. Five animals per group were used, divided as follows: Group 1 – composed of mice with intestinal inflammation induced by 10 days with water containing DSS 2.5% and receiving subcutaneous injections of saline 0.9%; Group 2 – mice under the same protocol of experimental colitis with DSS 2.5% and treated with subcutaneous injections of empty nanoparticles; Group 3 – mice under the same protocol of experimental colitis with DSS 2.5% and treated with subcutaneous injections of DHEA (40 mg/kg/day) emulsified in nanoparticles. The animals were weighed daily to monitor weight loss, in addition to observing the clinical signs such as moistened anus, diarrhea, bleeding, piloerection and hyperresponsiveness. Feed and water consumption were also monitored throughout the experimental period.

### *4.13 FITC-Dextran assay*

Food and water were withdrawn 24 hours before euthanasia of the animals, approximately 4 hours before euthanasia, FITC-dextran (PM 4000; FD4; Sigma-Aldrich, St Louis, MO) was administered via gavage at the concentration of 44 mg/100 g body weight. After this period, blood was collected and centrifuged at 5000 xg for 30

minutes and serum was collected. The fluorescence intensity was determined by reading the absorbance of the samples by spectrophotometry (excitation, 492 nm; emission, 525 nm). Serum FITC-dextran concentrations were determined using a standard curve generated by serial dilution of FITC-dextran.

### *4.14 Statistics*

The results found were tabulated in the Graphpad Prism 8.0.1 program, which was used to perform the statistical analyses. Shapiro-Wilk or Kolmogorov-Smirnov tests were used to test the data normality. Unpaired t test or Mann-Whitiney test were used according the data normality for two groups analysis. For t test Welch correction was applied when the variance was not homogeneous. For three or more groups ANOVA or Kruskal-Wallis test were used according the data normality. Friedman test was used to paired analysis for three or more groups. The observed differences were considered significant when $p < 0.05$.

### *4.15 Ethics*

The procedures with human recruitment and biological samples manipulation were approved by the ethics committee of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, under number 2.162.611 and the participants gave their written consent. All procedures were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (Appendix A).

All animals' experiments were in accordance with the ethical principles proposed by the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethics Committee on Animal Use of the Faculty of Pharmaceutical Sciences of

Ribeirão Preto, University of São Paulo (CEUA/FCFRP/USP), under number 19.1.1154.60.5 (Appendix B).

# 5 Results and Discussion

## 5. RESULTS and DISCUSSION

### *5.1 Sex steroid hormones influence the responsiveness to the anti-TNF therapy*

Considering that hormones are central regulators of the organic systems functions, the interaction between the endocrine and immune systems is of special importance for the homeostasis and human diseases pathophysiology.[120] Despite this, the involvement of the endocrine system in the regulation of immune responses is not yet fully understood, thus clearing these immunoendocrine interactions deserve to be better explored. It is known that the synthesis of hormones can occur and be influenced by immune cells and cytokines, on the other hand, the hormones produced by the endocrine glands can influence the function of innate and adaptive immunity, thus mediating the stimulation or inhibition of immunologic responses.[121]

This work is studying the effects of steroid hormones on the modulation of the immune response, thus we investigated whether there are differences in gene expression in intestinal biopsies between men and women diagnosed with CD. For this purpose, we performed differential gene expression analysis over samples of intestinal biopsies in a study deposited by Peters et al. 2017,[122] in Gene Expression Omnibus (GEO),[123] accession GSE100833. We focused in colons samples from inflamed and non-involved area were taken from patients non-responsive to anti-TNF before the next therapy strategy.

The gene expression variation between men and women was very similar, thus none clusters were visualized (Fig. 8A and 8B). In concordance, a few differentially expressed genes (DEGs), according an adjusted p-value < 0.05 and log2 fold change > 1.5, were observed and it was all the same for both conditions, non-involved and inflamed area (Fig. 8C and 8D). Only genes related to sex determination were among

the DEGs. The genes *TSIX* and *XIST* located on chromosome X of the pair 23 were upregulated on female samples, while *DDX3Y*, *EIF1AY*, *KDM5D*, *RPS4Y1*, *TXLNGY*, *USP9Y*, *UTY* and *ZFY* located on chromosome Y were upregulated on male samples.[124] Therefore, no DEGs were observed regarding genes related to immune response and to the steroidogenesis.

The next step was to investigate the endocrine axis genes expression such as, biosynthesis and metabolism of androgen and estrogen hormones (Fig. 2-4). Also, genes related to the receptors AR, ERα and ERβ were investigated (Fig. 9-11). The gene list was obtained from the MetaCore software (Clarivate™) and described in Table 2. To this end, it was analyzed the expression profile by array from a study deposited at GEO, accession GSE16879.[51] In this study, colon samples from CD patients were collected before and after 4-6 weeks of the treatment with Infliximab (IFX), in which includes 12 responder (RP) and 7 non-responders (N-RP) patients before the therapy and 11 RP and 7 N-RP after the IFX treatment. Also, 6 samples of healthy controls (HC) were taken. The samples from ileal CD and ulcerative colitis were not included in our analysis.

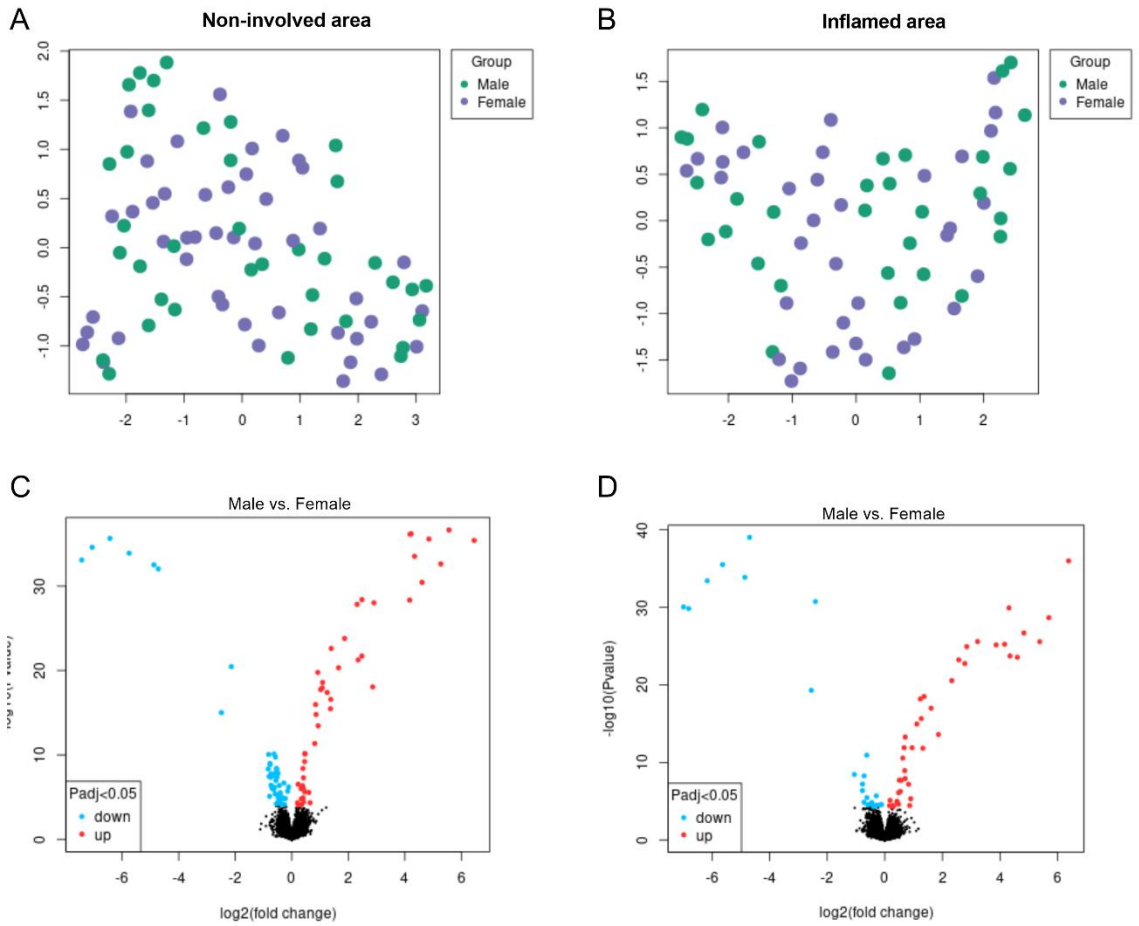


**Figure 8. Only genes related to sex determination are differentiated expressed between men and women in colon bulk transcriptome.** GSE100833 was analyzed on GEO2R[113, 114] using forced normalization and limma package v3.54.1.[112] The figures were also generated on GEO2R. To non-involved area 37 male and 45 female colon samples were analyzed, while to inflamed the number were 32 to male and 35 to female. Approximation and Projection (UMAP) in **(A)** non-involved and **(B)** inflamed area. Volcano plot showing the differentially expressed genes (DEGs) in **(C)** non-involved and **(D)** inflamed area. Uniform Manifold P-values were adjusted for multiple comparisons (adj. P-value < 0.05) using the Benjamini-Hochberg (BH) approach.[125]

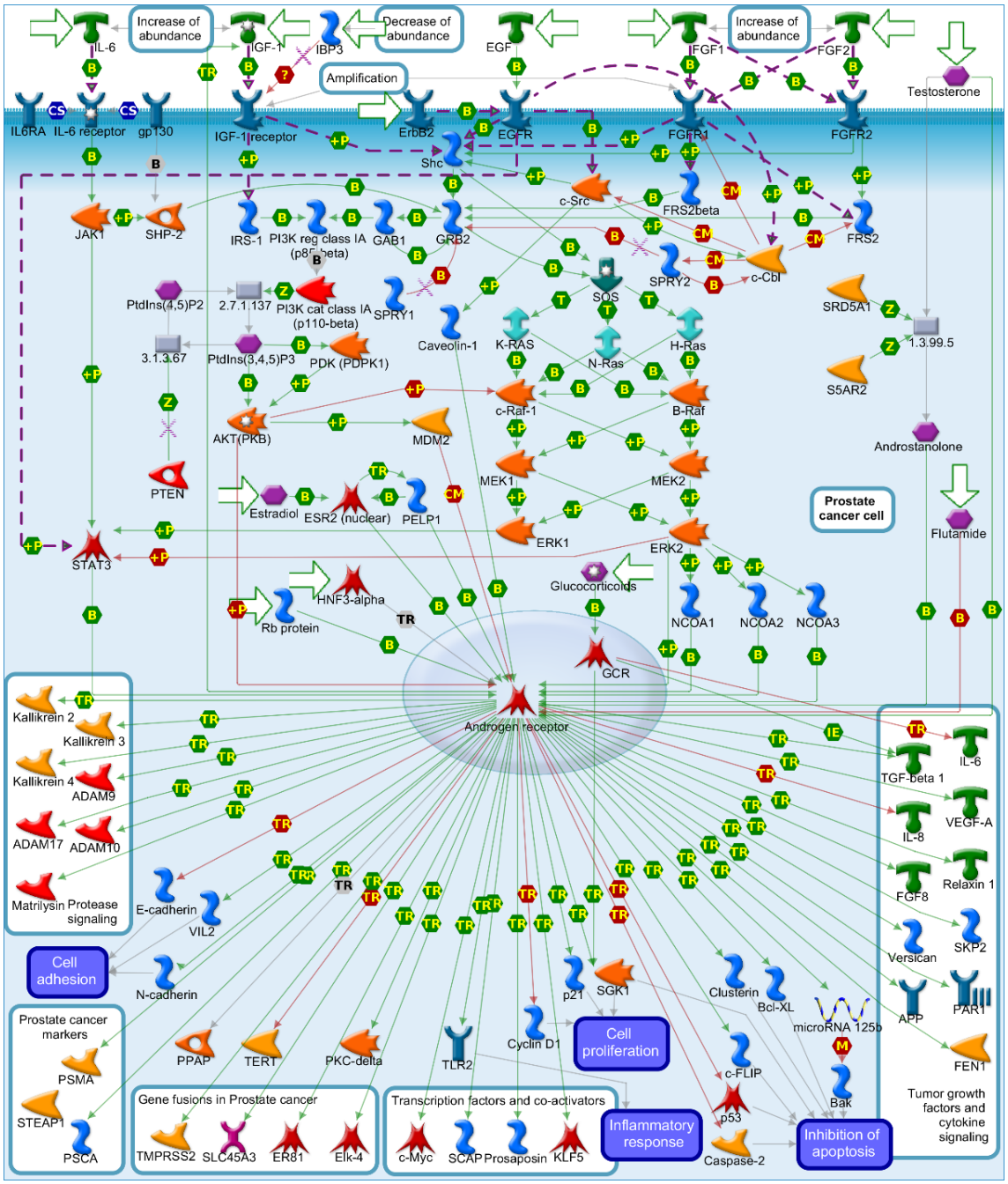


**Figure 9. Androgen receptor (*AR*) related genes.** Figure obtained from the MetaCore software (Clarivate™).

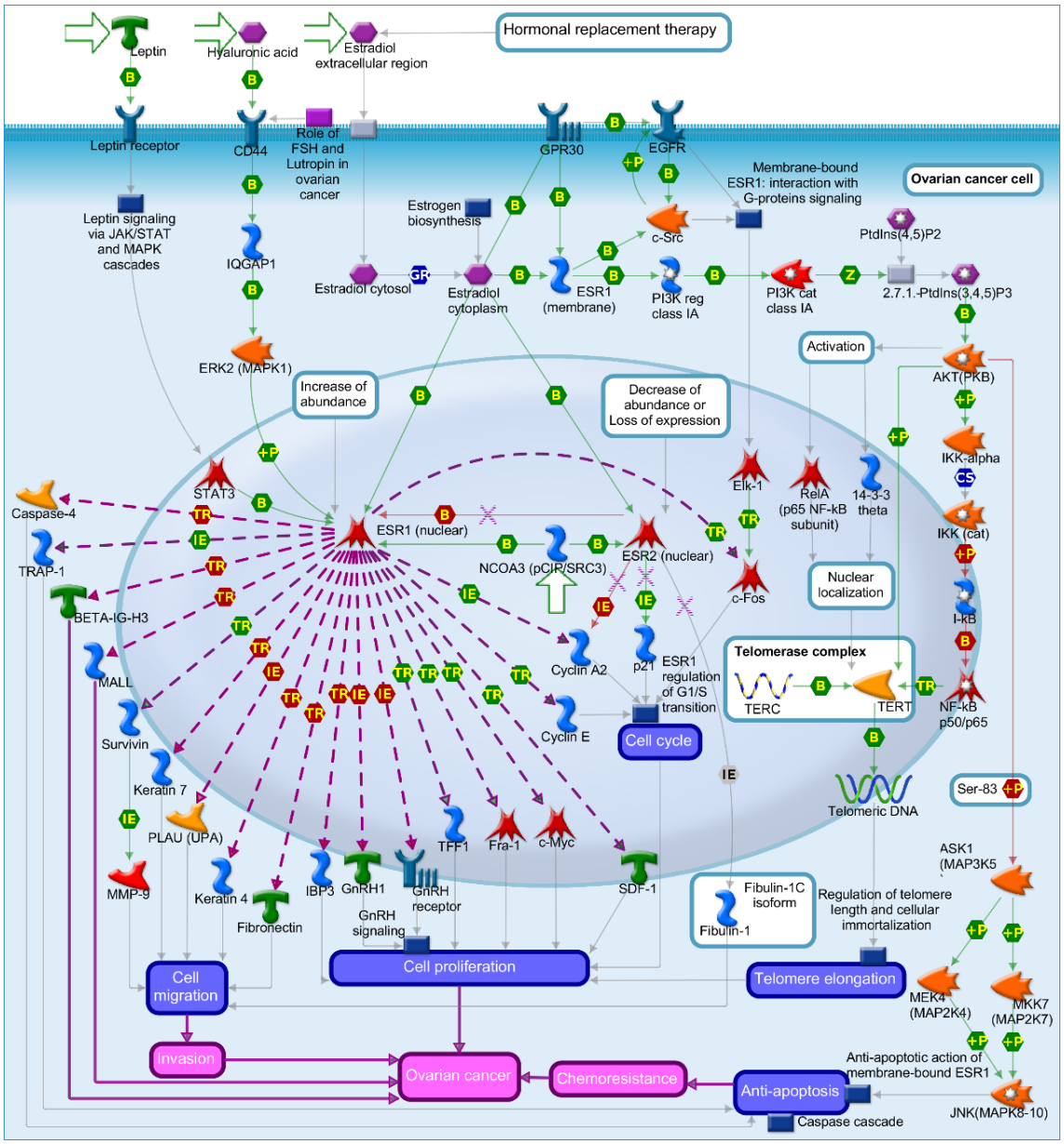


**Figure 10. Estrogen receptor alpha (*ESR1*) related genes.** Figure obtained from the MetaCore software (Clarivate™).

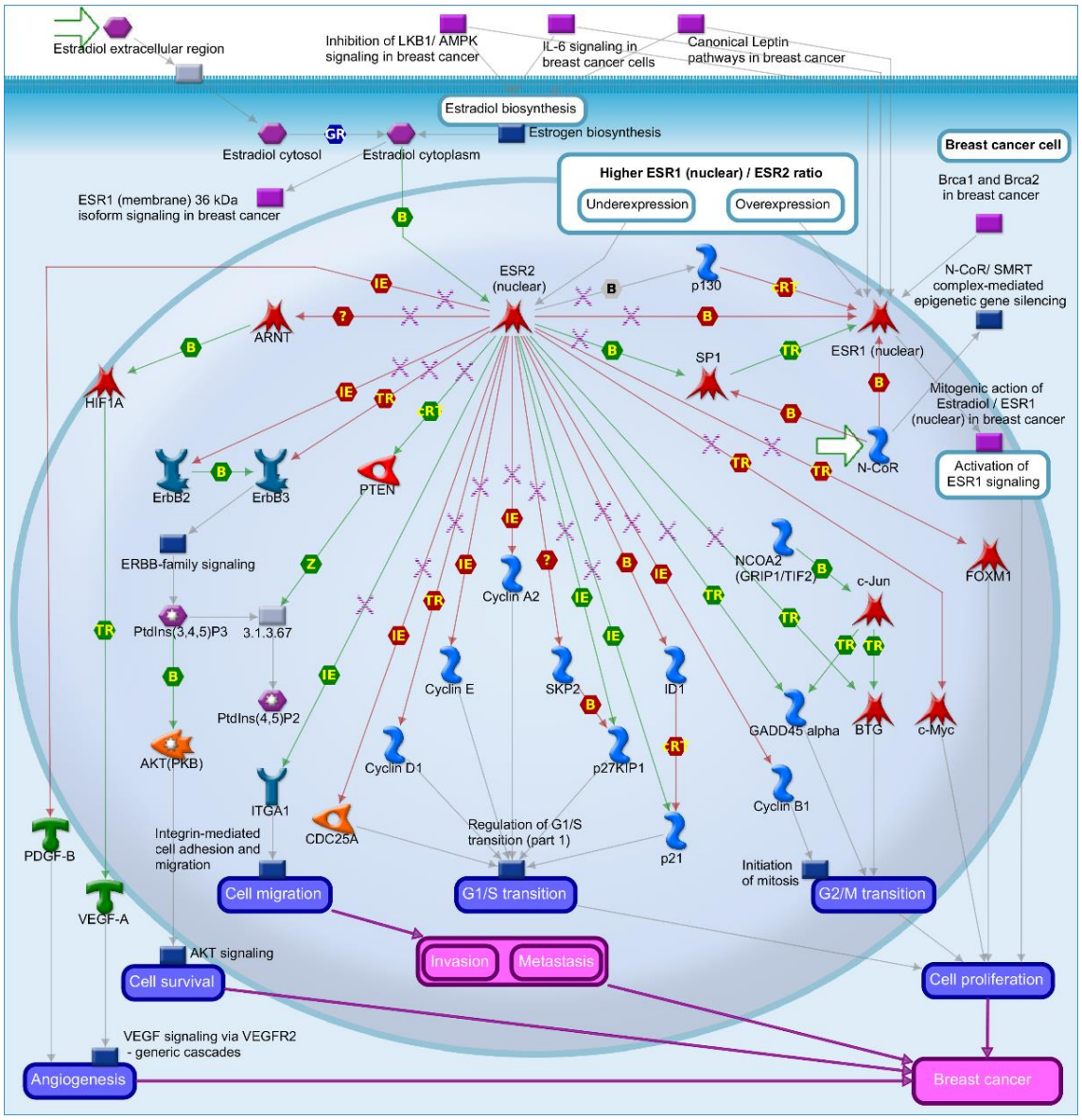


**Figure 11. Estrogen receptor beta (*ESR2*) related genes.** Figure obtained from the MetaCore software (Clarivate™).

First, it was conducted principal component analysis (PCA) to observe the variation on the global gene expression among the individuals. While the responder patients changed their transcriptional profile and became similar to the HC (Fig. 12A) there was no change at all on N-RP (Fig. 12B). Besides that, it was conducted an analysis that calculate a score of molecular degree of perturbation (MDP)[126] on the patients' groups using the control group as reference for non-perturbation (mean MDP score zero) based on the global gene expression. Also, perturbation was observed in colon samples from CD patients, corroborating the grouping observed in PCAs. Additionally, the MDP scores revealed that the perturbations were diminished in RP after the treatment, whereas were more accentuated in N-RP before the treatment that also do not change after the IFX therapy (Fig. 12C).

The sex of the recruited subjects was not discriminated on the study, however, it was considered that the bulk transcriptome profile between men and women are very similar, as showed on Fig. 8. However, the PCA and MDP analysis strengthens the premise that there are no differences between men and women, since the most impactful effect in the grouping of samples is the clinical status of the patients.

In agreement, the gene expression profile among RP, N-RP and HC showed that there are considerable differences on the transcriptional profile of RP patients before the treatment with IFX compared to the HC, as showed by the DEGs on the volcano plots (Fig. 13A), but these differences were attenuated after the IFX therapy (Fig. 13B). However, N-RP patients are not capable to reduce these differences after the treatment compared to HC (Fig. 13C and 13D).

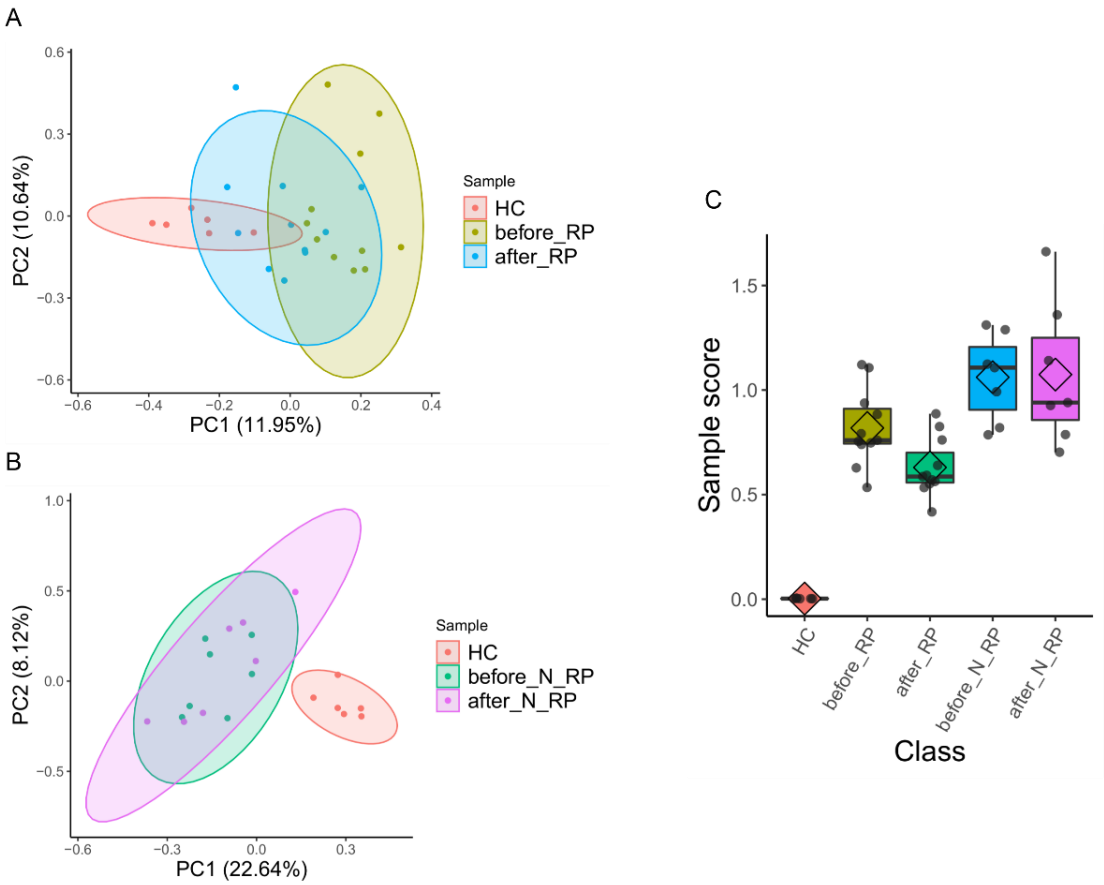


**Figure 12. Refractory patients do not change gene expression variation after the treatment.** Analysis based on the global gene expression. **(A,B)** Biplot showing the first two principal components obtained from Principal Component Analysis (PCA) using *prcomp* function of R. **(C)** Molecular degree of perturbation (MDP) score based on the top 25% genes with the highest variance using MDP package (version 3.16). The plots were constructed with ggplot2 package (version 3.3.5) with 95% confidence interval for ellipses.

In fact, there are differences on the transcriptional profile of the N-RP to the RP before the treatment (Fig. 14A), which is one of the probable explanations to the failure of IFX administration. Also, these differences are accentuated after the IFX (Fig. 14B), because the therapy is modulating the inflammation in RP and changing the gene expression (Fig. 14C). Moreover, the treatment is incapable of changing the transcriptional profile of the N-RP individuals with no DEGs observed after the treatment (Fig. 14D).

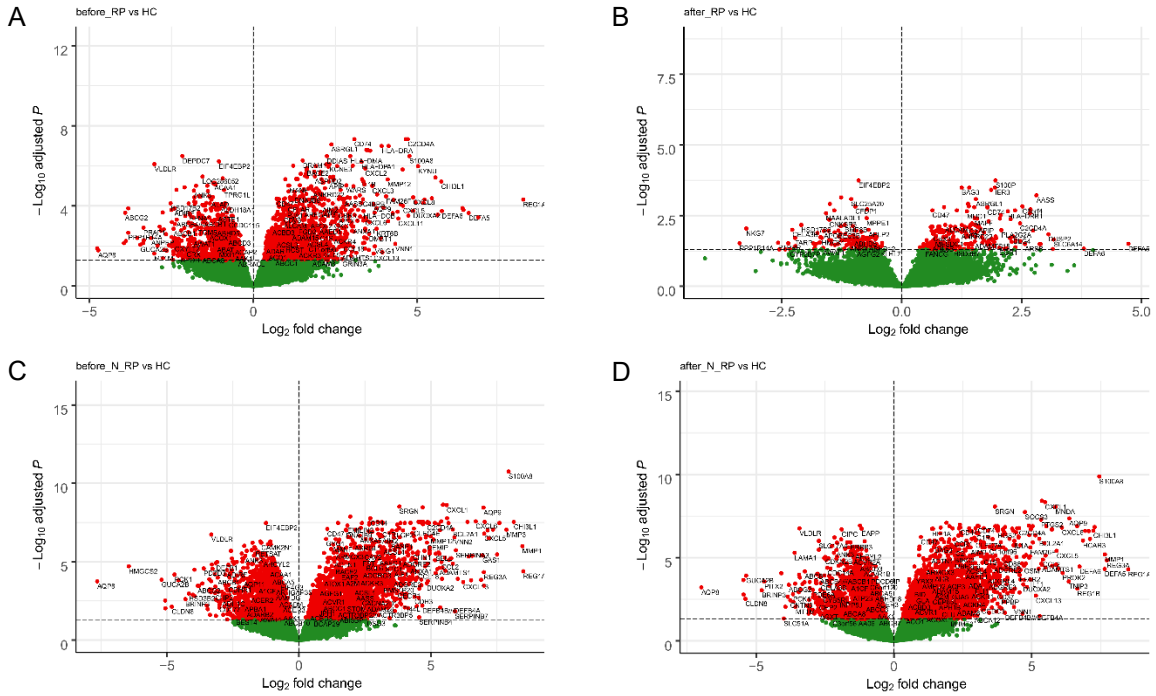


**Figure 13. Responder patients to IFX change their transcriptome profile in response to the treatment.** Volcano plots of differential gene expression analysis constructed using EnhancedVolcano R package (version 1.12.0). Responder patients (RP) compared with healthy controls (HC) **(A)** before and **(B)** after treatment. Non-responder patients (N-RP) compared with HC **(C)** before and **(D)** after treatment. P-values were adjusted for multiple comparisons using the BH approach and DEGs, indicated as red points, were defined as adj. P-value < 0.05.

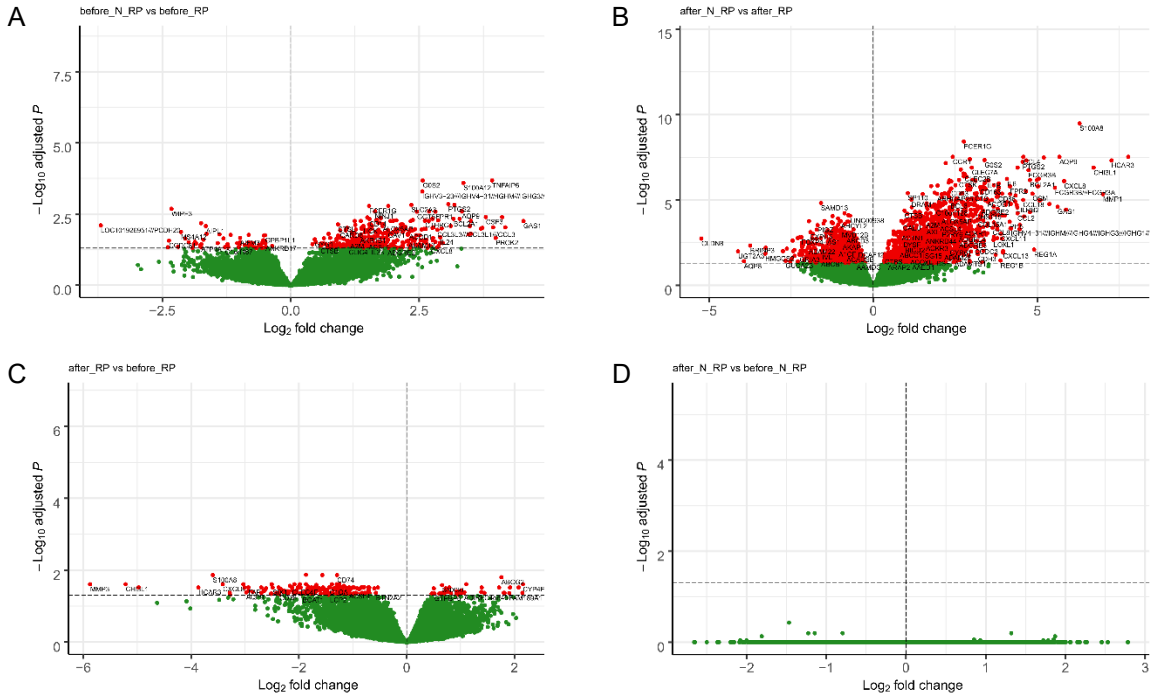


**Figure 14. Refractory patients to IFX does not change their transcriptome profile after the treatment.** Volcano plots of differential gene expression analysis constructed using EnhancedVolcano R package (version 1.12.0). Non-responder (N-RP) compared with responder patients (RP) **(A)** before and **(B)** after treatment. Comparisons of after versus before the treatment in **(C)** RP and **(D)** N-RP patients. P-values were adjusted for multiple comparisons using the BH approach and DEGs, indicated as red points, were defined as adj. P-value < 0.05.

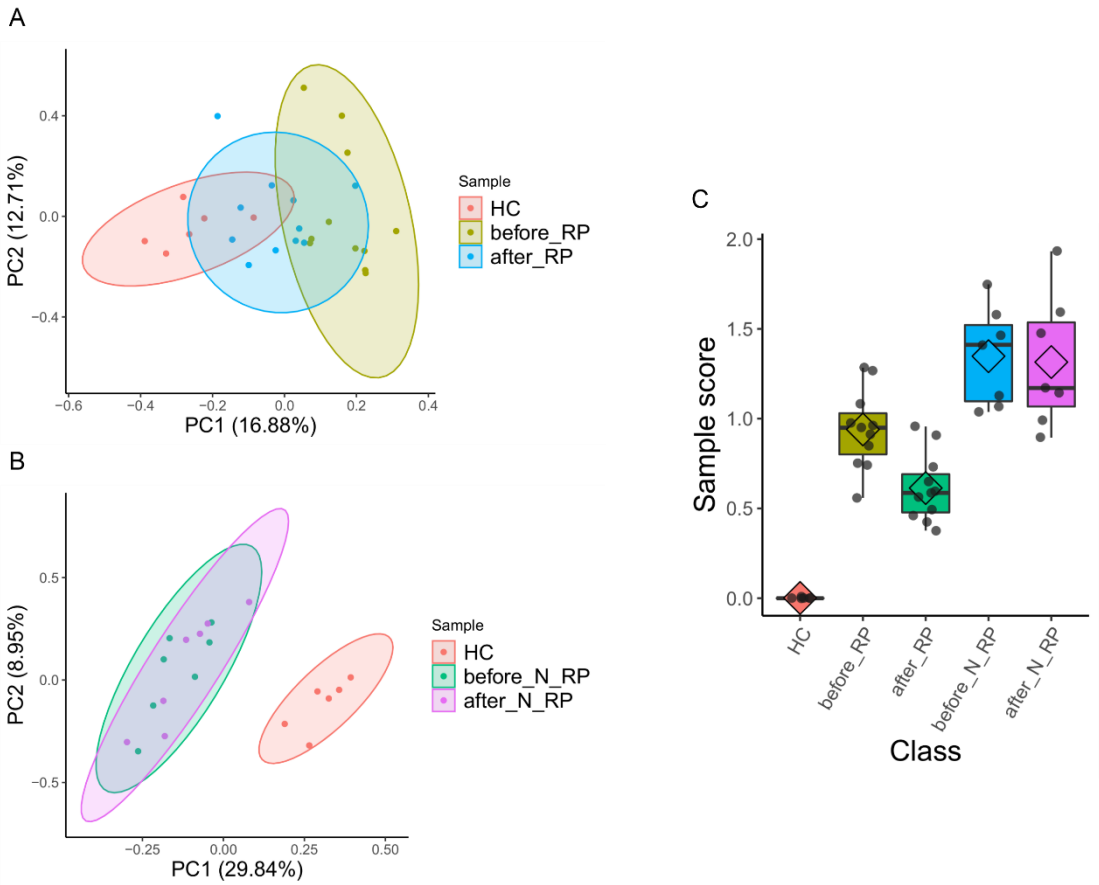


**Figure 15. The expression of endocrine genes follows the same pattern of the global gene expression.** Analysis based on the endocrine gene expression. **A,B)** Biplot showing the first two principal components obtained from Principal Component Analysis (PCA) using *prcomp* function of R. **(C)** Molecular degree of perturbation (MDP) score based on the top 25% genes with the highest variance using MDP package (version 3.16). The plots were constructed with ggplot2 package (version 3.3.5) with 95% confidence interval for ellipses.

Next, new PCAs and MDPs analysis were conducted, but only considering the subset of genes related to the "endocrine axes" such as, "androgens biosynthesis and metabolism", "estrogens biosynthesis and metabolism", "androgen receptor signaling", "estrogen receptor alpha signaling" and "estrogen receptor beta signaling" (Fig. 15A-C). Therefore, the transcriptomic profile and perturbation score observed on the groups followed the same variation of the global gene expression observed in Fig. 12.

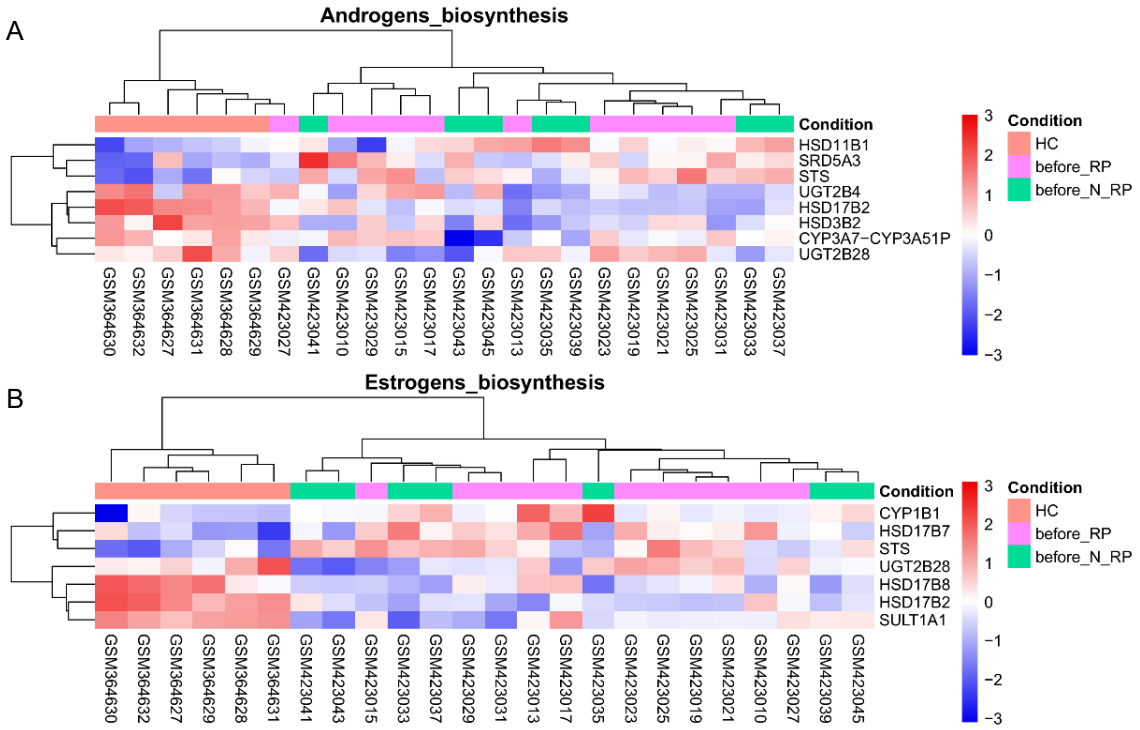


**Figure 16. The metabolization and inactivation of sex hormones are upregulated in healthy controls.** Heatmaps showing the differentially expressed genes (DEGs) per individual with ward.D2 clustering method. **(A)** Androgen synthesis and metabolism. **(B)** Estrogen synthesis and metabolism. Only genes with adj. p-value < 0.05 were considered. The heatmaps were generated with the package pheatmap (version 1.0.12).

In addition, the androgens and estrogens biosynthesis and metabolism pathways (MetaCore – Clarivate™) were investigated on the patients before the IFX therapy and control samples with the goal to determinate some prediction of response to the treatment based on these endocrine axes. Both pathways were capable to discriminate the HC to the CD patients, as the heatmaps organized the subjects according the similarities in expression profiles of genes in theses axes. However, they were not capable to distinguish the patients based on the responsiveness to the IFX (Fig. 16A and 16B). In general, in the colonic samples from HC, compared to CD patients, the expression of enzymes that favor the biosynthesis and activation of hormones were downregulated, such as *SRD5A3*, *STS* and *CYP1B1*.[65] Moreover, the expression of enzymes that favor the metabolization and inactivation of androgens

and estrogens was upregulated, such as *UGT2B4*, *UGT2B28*, *SULT1A1*, *HSD17B2* and *HSD17B8*.[65, 127]

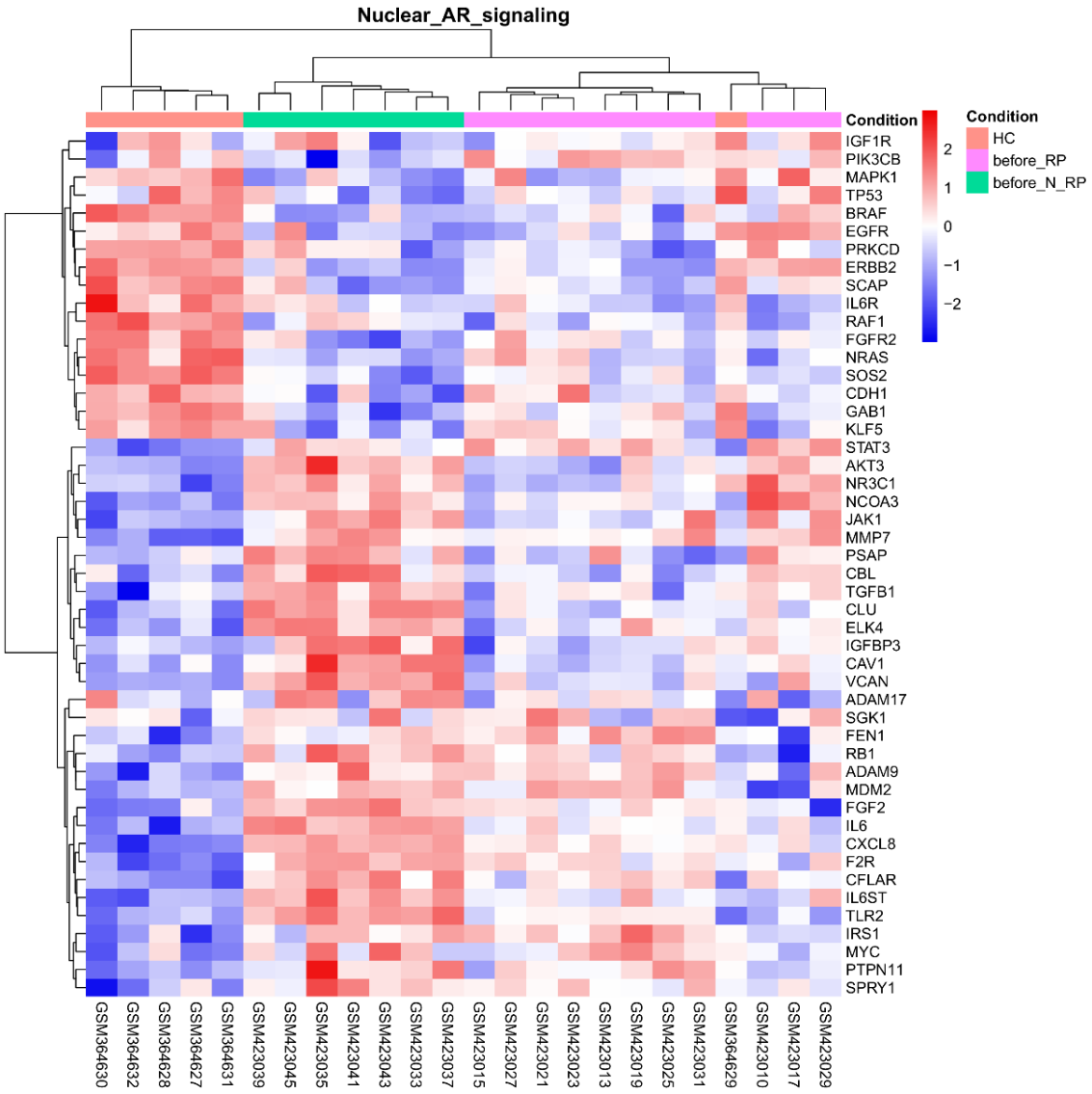


**Figure 17. Non-responder patients have a unique androgen receptor signaling pathway expression.** Heatmap showing the differentially expressed genes (DEGs) per individual with ward.D2 clustering method for androgen receptor signaling pathway. Only genes with adj. p-value < 0.05 were considered. The heatmaps were generated with the package pheatmap (version 1.0.12).

Next, the pathways related to the androgens and estrogens receptors were also investigated. Regarding the AR and ERα signaling pathways, the samples were divided in two big clusters separating HC from CD. In addition, the patients cluster was subdivided in another two clusters, which clearly separated the RP from N-RP patients. Therefore, the N-RP patients have an opposite gene expression profile to the

HC, while the RP have transcriptional profiles intermediate between HC and N-RP (Fig. 17 and Fig. 18). Nonetheless, the ERβ signaling pathway differentiated the HC from all the CD patients, but RP and N-RP have a similar gene expression profile (Fig. 19).

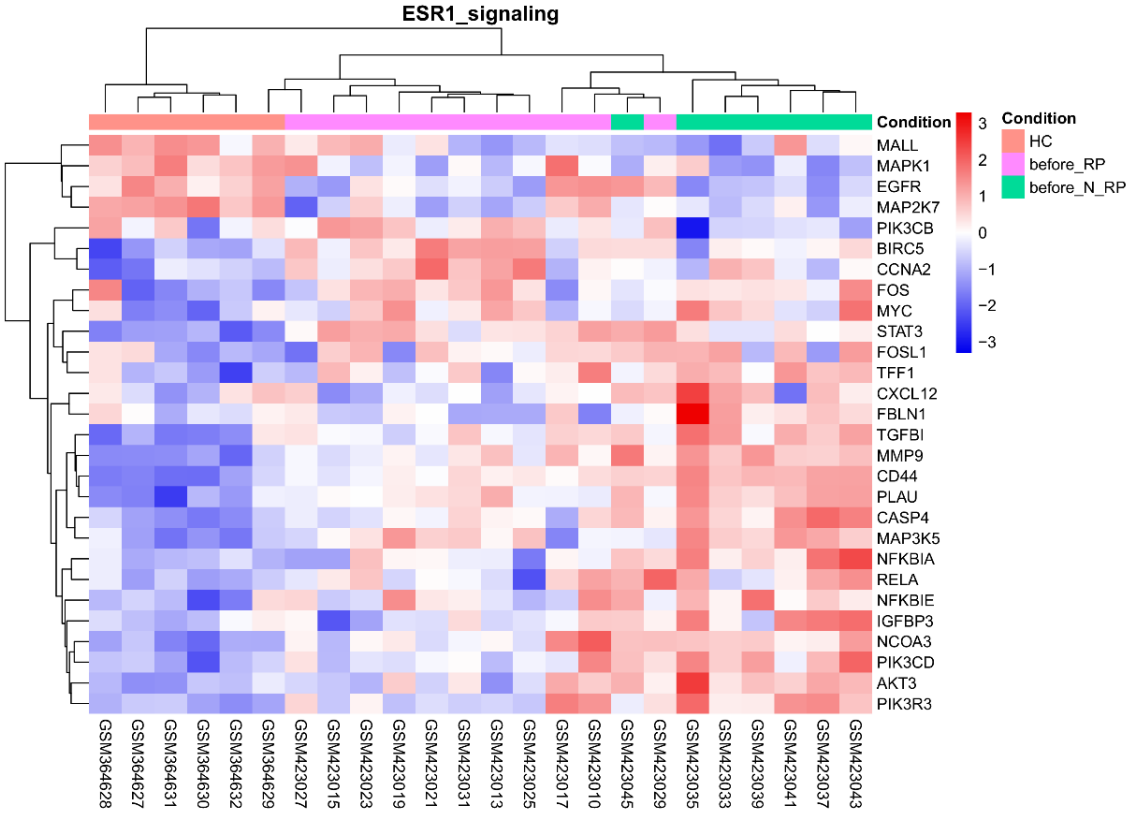


**Figure 18. Non-responder patients have a unique estrogen receptor alpha signaling pathway expression.** Heatmap showing the differentially expressed genes (DEGs) per individual with ward.D2 clustering method for estrogen receptor alpha signaling pathway. Only genes with adj. p-value < 0.05 were considered. The heatmaps were generated with the package pheatmap (version 1.0.12).

Considering that the genes downstream to the AR, ERα and ERβ signaling are involved in others molecular processes, it was investigated which pathways are been activated in HC and CD, especially the N-RP. Thus, the genes downregulated in CD, but enriched in HC, and the upregulated in CD were submitted separately to Enrichr software[128, 129, 130] in order to identify the enriched pathways on Reactome and the ontologies on GO Biological Process. The top 10 axes in each analysis are described on

Fig. 20. In HC, the most pathways upregulated, in response to AR, ERα and ERβ signaling axes, were involved in MAPK cascade and ERBB signaling pathways (Fig. 20A). Both are related to a mitogen function and regulating the cell cycle and proliferation.[110, 131] On the contrary, the most exacerbated pathways in CD, especially the N-RP, were related to immune response and inflammation. The interlekin-6 signaling pathway was the most highlighted axis upregulated in CD (Fig. 20B).

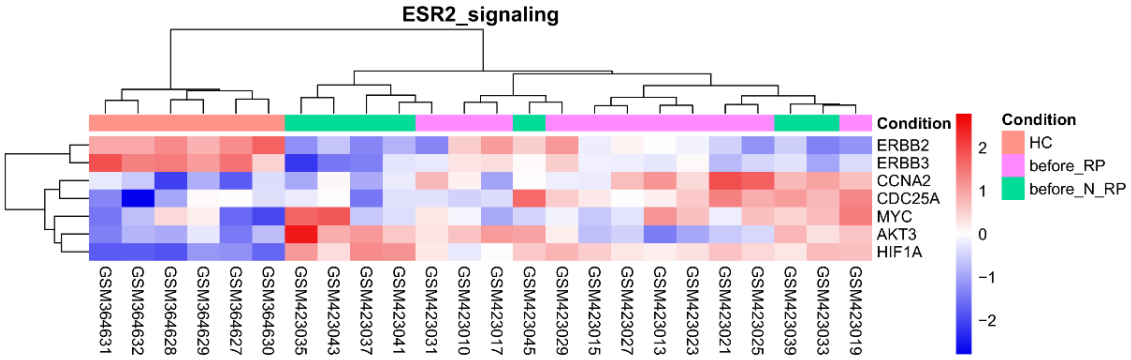


**Figure 19. Estrogen receptor beta signaling pathway expression can differentiate healthy controls to patients. H**eatmap showing the differentially expressed genes (DEGs) per individual with ward.D2 clustering method for estrogen receptor beta signaling pathway. Only genes with adj. p-value < 0.05 were considered. The heatmaps were generated with the package pheatmap (version 1.0.12).

Pubertal growth is tightly related to sex steroid hormones actions, which will coordinate several bodily functions improving, sex maturation, aging, significant changes in the organic systems and adult phenotype development.[132] Our results pointed that adult CD patients are under sex hormones unbalanced conditions. Thus, we can expect that pubertal adolescents diagnosed with IBD can also present sex steroid hormone disfunction. In fact, delays in pubertal growth in pediatric IBD patients is well documented, such as impaired bone age,[133, 134] late menarche,[133] delayed breast development and testicular enlargement.[135] Among sex, CD and UC, boys with CD are more likely to develop stronger disorders in pubertal growth.[136] Even when the disease is under control these delays in puberty persist,[137] although the

treatment with anti-TNF increases serum levels of testosterone, estradiol and gonadotropins in adolescents.[138]

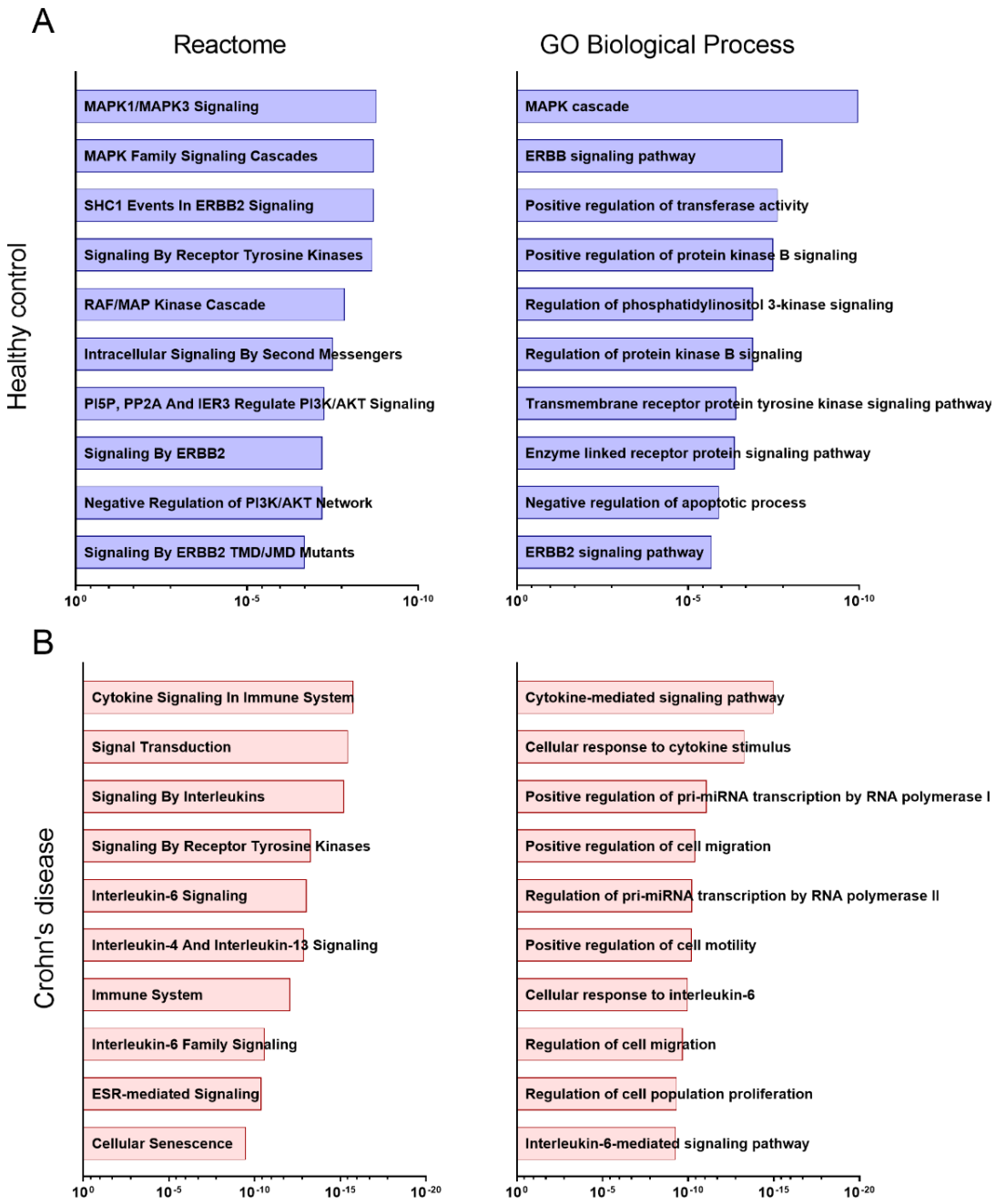


**Figure 20. In response to AR, ERα and ERβ, IL-6 signaling pathway are upregulated in Crohn's disease.** Bar plots showing the enriched top 10 pathways on Reactome and also the top 10 ontologies on GO Biological Process. Analysis made by Enrichr software for DEGs. **(A)** healthy controls and **(B)** Crohn's disease patients. Only axes with adj. p-value < 0.05 were considered. The bar plots were generated on GraphPad Prism v.7.00.

Furthermore, elderly hypogonadal men diagnosed with CD when supplemented with testosterone have significant improvement in Crohn's disease activity index. Also, the normalization of testosterone has capacity to dimmish the C-reactive protein (CRP).[139, 140] On the other hand, women in use of oral contraceptive (OCP) are at higher risk of IBD development, 30% increased risk for UC and 24% for CD.[141] The progression of the disease is also intensified by the use of OCP, affecting even the risk of bowel surgeries.[142, 143]

In response to endogenous sex hormones, the receptors AR, ERα and ERβ are activated regulating several genes expression. However, the upregulated genes downstream to these receptors are different between healthy individuals and CD patients, especially the non-responders to anti-TNF therapy. The mitogen-activated protein kinases (MAPKs) were the most highlighted pathway in healthy controls, which was expected since this pathway is involved in cell proliferation, differentiation, migration, survival and apoptosis.[144] MAPKs is highly associated with tumors, since its activation increases cell proliferation. Well, most of the discovers about AR, ERα and ERβ were made in cancer cell lineages. Thus, we have to considerate that these receptors are already involved in regulating the cell cycling in homeostasis, besides a more prominent role in cancer.[145, 146]

The receptor ERBB2, also known as HER2 (human epidermal growth factor receptor 2), is part of the epidermal growth factor (EGF) family. Like the MAPK pathway, ERBB2 signaling was upregulated in HC compared to CD as well. As described in its name, ERBB2 is involved in cell proliferation and atypical activation of this receptor can lead to uncontrolled cell growth and cancer.[147, 148] In addition,

ERBB2 can activate MAPKs pathway to cell proliferation activation, mainly under ERα and ERβ activation.[149]

In contrast, the genes up-regulated in CD patients were involved in inflammation-related pathways, mainly IL-6 axis. Indeed, IL-6 is increased in CD even more than in UC and HC. Furthermore, among CD patients, IL-6 tends to be higher in colonic disease than in ileal CD.[150] Whether serum IL-6 is related to disease activity remains unclear and data are controversial.[151] In addition, this cytokine seems to have a dual role in the development of IBD.[32, 38] While it can enhance the integrity of the intestinal barrier and protect the intestinal epithelial cells (IECs) from pathogens, it can also promote inflammation by activating immune cells like macrophages, dendritic cells, and T lymphocytes.[32, 38] Moreover, IL-6 has been associated with promoting T cell proliferation and inflammation in the gut.[152] Despite this, blocking IL-6 has shown positive outcomes in refractory CD patients who show no response to anti-TNF therapy in clinical trials - ANDANTE I and II. However, the treatment may lead to complications, such as abscesses and perforations, which may be due to the impaired wound-healing function of IL-6 resulting from anti-IL-6 immunotherapy.[153]

### *5.2 Circulating DHEA is decreased in CD*

The bioinformatic analysis showed a hormonal dysregulation on CD patients, especially on those refractories to anti-TNF biological, which demands furthers investigations. For this end, it was recruited 39 CD patients from the IBD Outpatients Clinics of the Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto (HCFMRP/USP) and 20 HC individuals from the same institution. All the patients were male and under IFX therapy. Moreover, 61.54% were also treated with Azathioprine and 38.48% had active disease. The patients have no other comorbidities disease or condition.

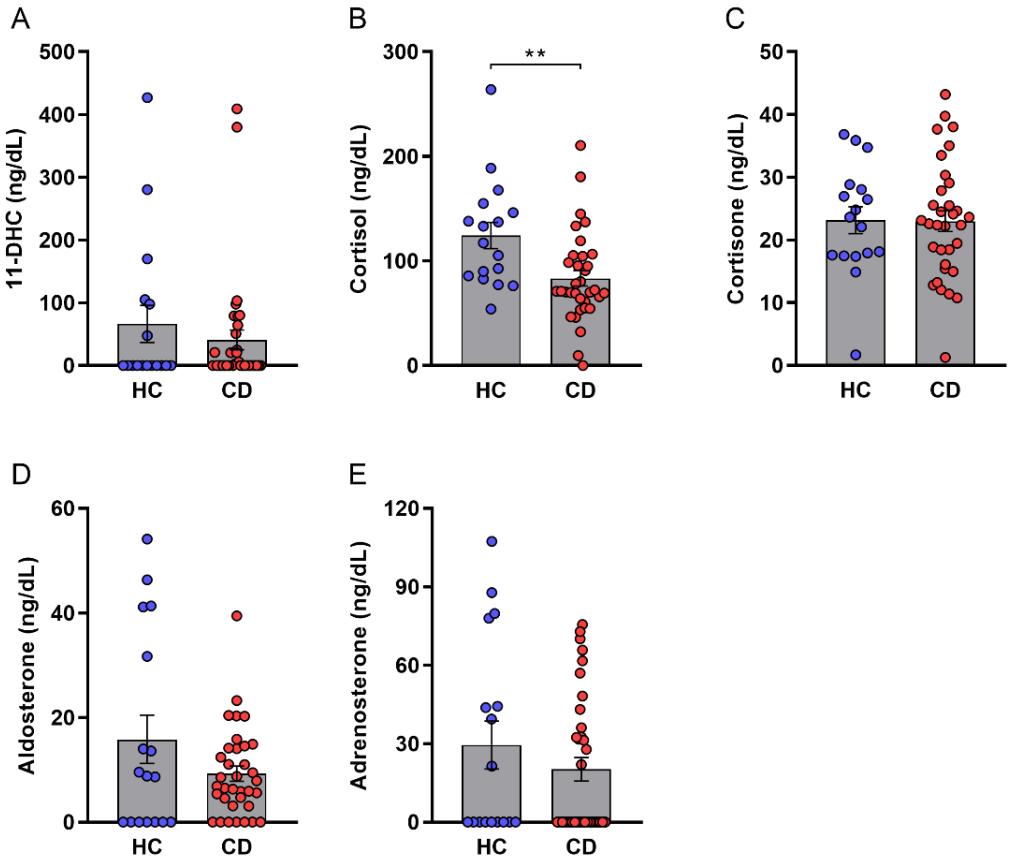


**Figure 21. Cortisol hormone is reduced in Crohn's disease.** Steroid hormones were measured by mass spectrometry (LC-MS/MS) on plasma samples. Blue dots are representing the healthy controls (HC), while the red dots the Crohn's disease patients (CD). **(A)** 11-DHC; **(B)** Cortisol; **(C)** Cortisone; **(D)** Aldosterone; **(E)** Adrenosterone. The results are expressed nanograms per deciliter (ng/dL) with mean and standard error of the mean. *$p < 0.05$; **$p < 0.01$, using the unpaired t test or Mann-Whitney according the data normality.

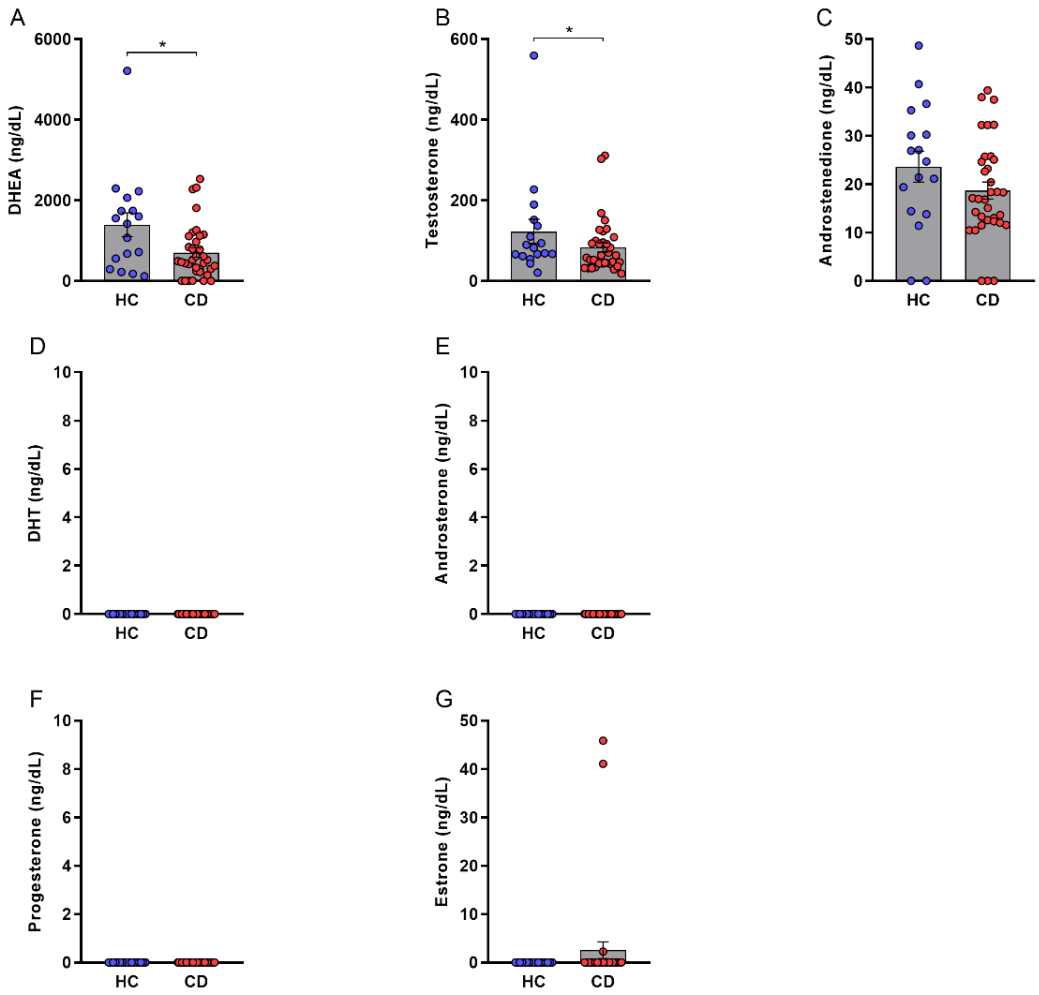


**Figure 22. Cortisol hormone is reduced in Crohn's disease.** Steroid hormones were measured by mass spectrometry (LC-MS/MS) on plasma samples. Blue dots are representing the healthy controls (HC), while the red dots the Crohn's disease patients (CD). **(A)** DHEA; **(B)** Testosterone; **(C)** Androstenedione; **(D)** Dihydrotestosterone; **(E)** Androsterone; **(F)** Progesterone; **(G)** Estrone. The results are expressed nanograms per deciliter (ng/dL) with mean and standard error of the mean. *$p < 0.05$, using the unpaired t test or Mann-Whitney according the data normality.

Thereby, it was measured several hormones in plasma samples by mass spectrometry (LC-MS/MS) to accesses this unbalanced hormonal finds. First, it was looked at the endogenous glucocorticoids, such as 11-dehydrocorticosterone (11-DHC), cortisol and cortisone (Fig. 21A-C). Also, adrenal hormones, such as aldosterone and adrenosterone (Fig. 21D and 21E). Among these hormones, only cortisol was reduced in CD patients (Fig. 21B). However, although patient and control

samples were collected in the same time slot, this collection was not strictly rigorous for cortisol due to the management of patients in the hospital.

Concerning the androgens hormones, the dehydroepiandrosterone (DHEA) and testosterone (Fig. 22A and 22B) were decreased in CD patients. No differences in androstenedione. Further, it was measured the testosterone metabolites dihydrotestosterone (DHT) and androsterone (Fig. 22D and 22E), also the hormones related to the female sex progesterone and estrone (Fig. 22F and 22G). Mostly of the individual has no detection for these hormones (Fig. 22D-G).

One of the aims of this study it is propose a hormonal therapy to regulate the exacerbating inflammation observed in Crohn's disease. Of course, not as main therapy, but an adjuvant treatment to be add together with immunobiologicals or immunosuppressants. Thus, considering that the DHEA is reduced in Crohn's disease, previous reports already corroborate this find,[154, 155] that women well tolerate DHEA supplementation since his androgenic effect is very low,[156, 157] that there are many other studies showing the immunoregulatory potential of DHEA[158, 159, 160] and the results of this thesis, we chose to continue our investigations focusing on the hormone DHEA. The following analysis strategies to investigate de DHEA is depicted in Fig. 23.

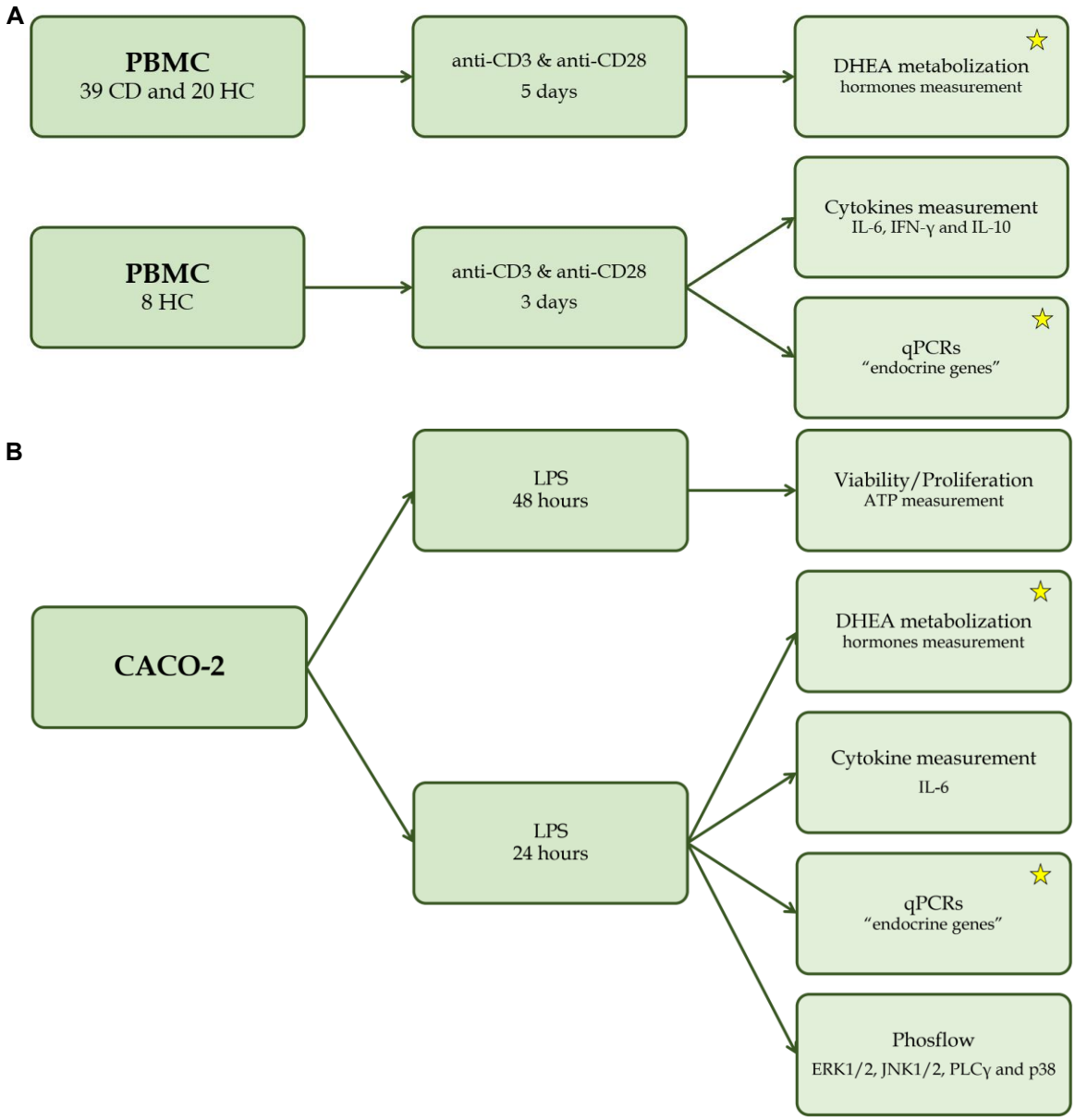


**Figure 23. DHEA mechanism of action investigation strategy. (A)** First, PBMC ($5x10^5$ cells/well) from 39 CD and 20 HC induviduals were stimulated *in vitro* with anti-CD3 and anti-CD28 (1.0 µg/mL) on presence or absence of DHEA 5.0 µM by 5 days. The suppernant were stored at -80ºC to cytokines hormones measurement. After, others 8 HC were recruited to investigate the DHEA mechanism of action. The PBMC ($5x10^5$ cells/well) were stimulated *in vitro* with anti-CD3 and anti-CD28 (1.0 µg/mL) on presence or absence of DHEA 5.0 µM and several antagonists by 5 days. The exacly conditions for the PBMC culture is detaild on Fig. 24. The suppernant were stored at -80ºC to cytokines measurement and the cells were kept in TRIzol™ to RNA extraction. **(B)** CACO-2 cells ($2x10^5$ cells/well) were stimulated *in vitro* with LPS (100 ng/mL) on presence or absence of DHEA 5.0 µM and several antagonists by 24 hours. The exacly conditions for the CACO-2 culture is detaild on Fig. 24. The suppernant were stored at -80ºC to cytokines and hormones measurement. The cells were kept in TRIzol™ to RNA extraction or had their proteins extracted to Phosflow performing. All the experiments have already been done, but the yellow star marks the analyzes that will still be carried out after the defense of this thesis.

We already knew that the PBMC from CD patients is more responsive to DHEA supplementation *in vitro* and this hormone can decrease the production of several inflammatory cytokines, such as TNF, IL-6, IL-1β, IFN-γ and IL-17, also IL-10 (data not publish yet). Thus, peripheral blood mononuclear cells (PBMC) from these individuals were isolated and simulated with anti-CD3 and anti-CD28 antibodies by five days in absence or presence of DHEA. Therefore, the aim is to observe DHEA metabolism in the culture supernatant by mass spectrometry between HC and CD; Are they different? This assay was able to identify DHEA metabolites, such as testosterone, androstenedione, DHT, androsterone and also estrone (Fig. 25). To date of the writing of this thesis, only samples from four patients were analyzed. We expect to finalize these experiments very soon.

| Map | Culture conditions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| PBMC culture | DMSO | DHEA | EST | EST<br>DHEA | EST<br>DHEA<br>FULV | EST<br>DHEA<br>FLUT | EST<br>DHEA<br>FLUT<br>FULV | EST<br>DHEA<br>FLUT<br>MPP | EST<br>DHEA<br>FLUT<br>PHTPP |
| CACO-2 culture | DMSO | DHEA | LPS | LPS<br>DHEA | LPS<br>DHEA<br>MPP<br>PHTPP | LPS<br>DHEA<br>FLUT | LPS<br>DHEA<br>FLUT<br>MPP<br>PHTPP | LPS<br>DHEA<br>FLUT<br>MPP | LPS<br>DHEA<br>FLUT<br>PHTPP |
| Receptors to be blocked | | | | → | ERs | AR | AR + ERs | AR + ERα | AR + ERβ |

**Figure 24. Culture map scheme.** DMSO was used as the drugs vehicle. The peripheral mononuclear cells (PBMC) or CACO-2 were supplemented with DHEA. Antibodies anti-CD3 and anti-CD28 were used as stimuli (EST) to PBMC and lipopolysaccharide (LPS) to CACO-2 cells. Flutamide (FLUT), Fulvestrant (FULV), MPP and PHTPP were used to block the androgen receptor (AR), estrogen receptors (ERs), estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), respectively. FULV was used to block ERs only in the PBMC cultures, to CACO-2 culture the combination of MPP+PHTPP was preferred.

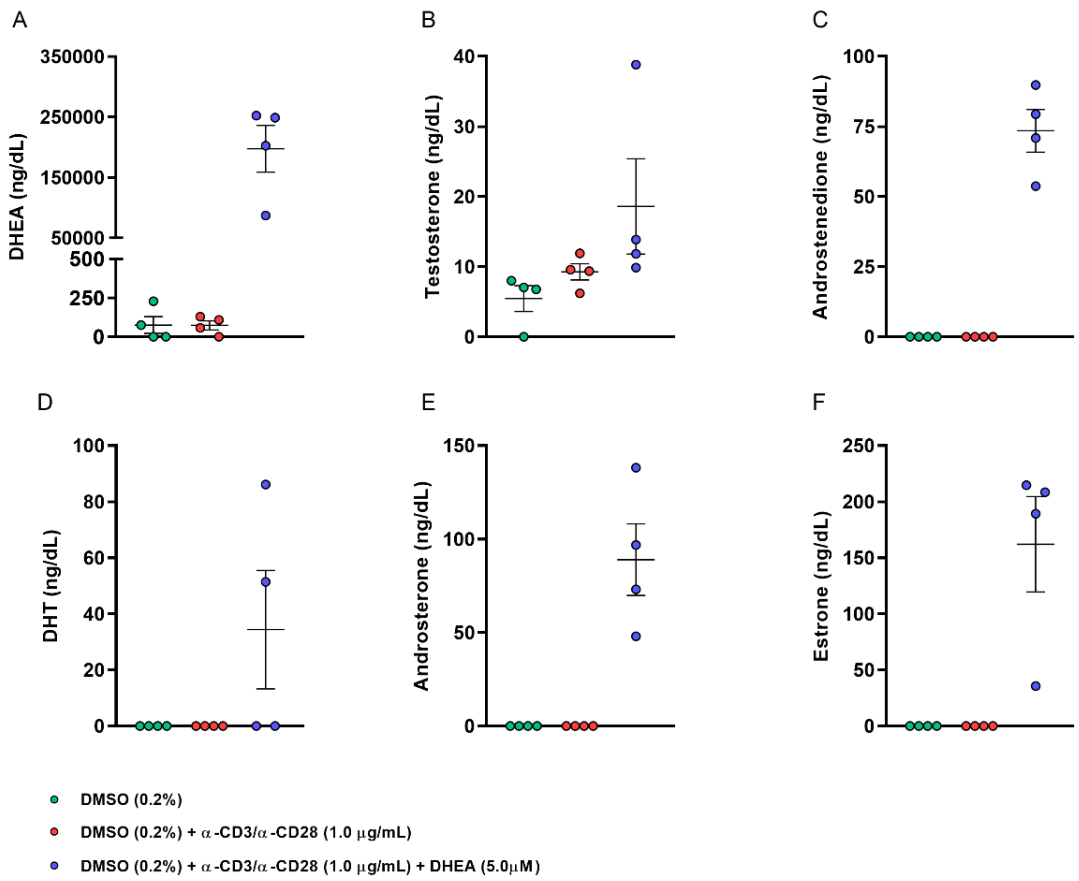


**Figure 25. DHEA is metabolized by patients' PBMC.** Steroid hormones were measured by mass spectrometry (LC-MS/MS) on supernatant samples. **(A)** DHEA; **(B)** Testosterone; **(C)** Andorstenedione; **(D)** DHEA; **(E)** Adrenosterone; **(F)** Estrone. The results are expressed nanograms per deciliter (ng/dL) with mean and standard error of the mean.

In order to block the endocrine axes, several combinations of the antagonists were used, as described in Fig. 24. The supernatant from the PBMC culture were kept to cytokines measurement such as, IL-6, IFN-γ and IL-10. DHEA *in vitro* supplementation diminished the production of the three cytokines measured, IL-6, IFN-γ and IL-10 (Fig. 26 A-E). The PBMC restored the capacity of IL-6 and IFN-γ production only when the AR and ERβ were antagonized (condition 9), suggesting the DHEA may partially uses the ERβ for its effects (Fig. 26E). No differences were found on the PBMC supplemented with DHEA only, but not stimulated (condition 2), compared to the condition 1 (data not shown).

Moreover, the PBMC and CACO-2 from these cultures were kept in TRIzol™ to RNA extraction and cDNA confection. The next step is to perform qPCR to several endocrine genes to help us define a pathway that DHEA is acting. Will be quantified the receptors genes *AR*, *ESR1* and *ESR2*. Other genes were chosen according the mostly significant genes expressed in the colon samples, that participate to the steroidogenesis, from the GSE16879 analysis (Fig. 16A and 16B). Also, the key genes enzymes for the steroid hormone biosynthesis and metabolism, such as:

Androgen biosynthesis:

- *HSD3B1* – DHEA → androstenedione; androstenediol → testosterone;
- *HSD17B3* – DHEA → androstenediol; androstenedione → testosterone;
- *SRD5A1* and *SRD5A2* – testosterone → DHT;
- *STS* – DHEA sulfate → DHEA; androstenediol sulfate → androstenediol.

Estrogen biosynthesis:

- *ARO* – androstenedione → estrone; testosterone → estradiol;
- *HSD17B7* – estrone → estradiol;
- *STS* – estrone sulfate → estrone.

Sex hormones inactivation and metabolism:

- *HSD17B7* – DHT → androstenediol;
- *HSD17B2* – androstenediol → DHT; testosterone → androstenedione; estradiol → estrone;
- *SULT1A1* – estradiol → estradiol sulfate;
- *CYP1B1* – estradiol → 4-hydroxyestradiol;
- *UGT2B28* – glucuronidation of several hormones.

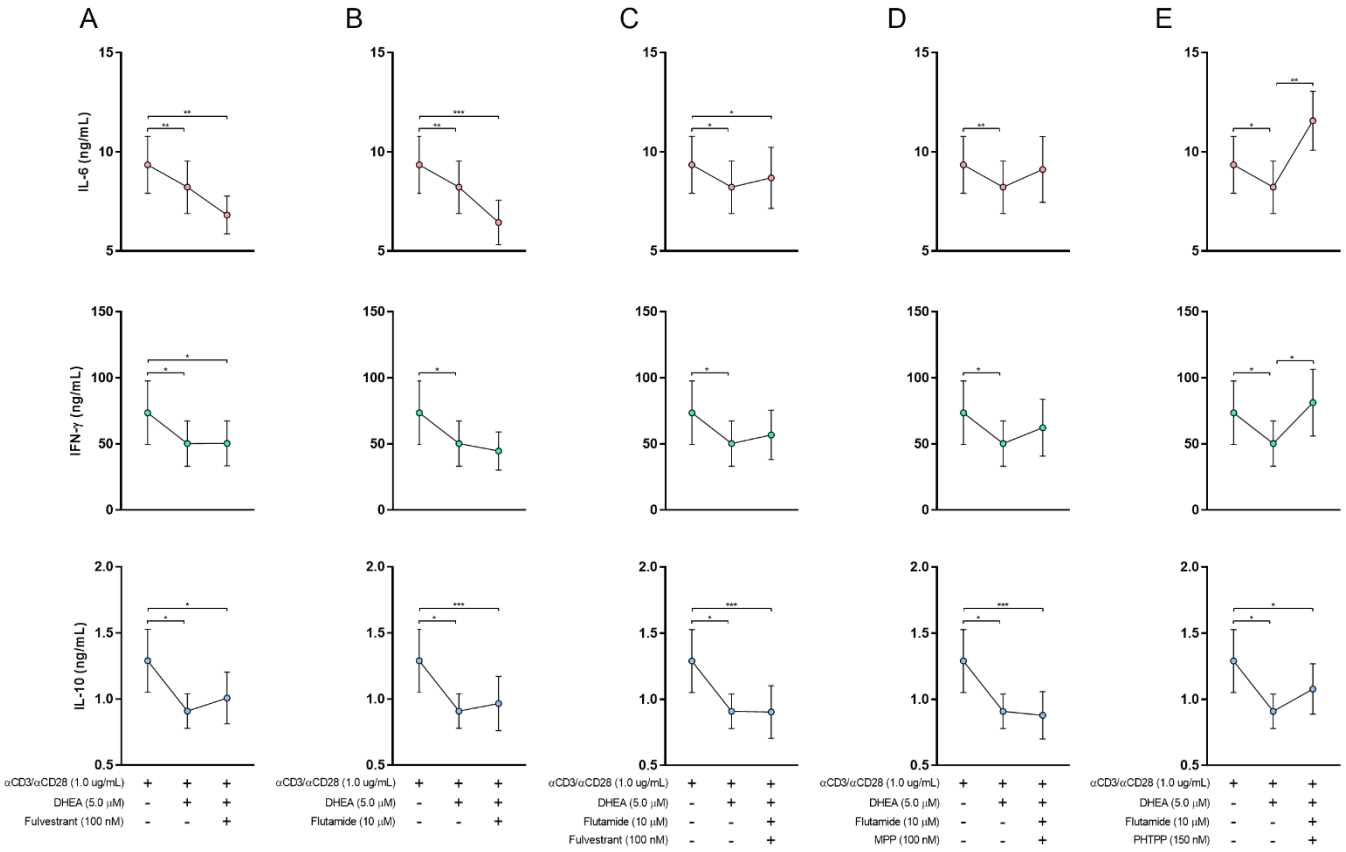


**Figure 26. DHEA may use ERβ to exert its regulatory effects.** The cytokines IL-6, IFN-γ and IL-10 were measured by immunoenzymatic assay. The following receptors were blocked: In **(A)** ERs; **(B)** AR; **(C)** AR + ERs; **(D)** AR + ERα; **(E)** AR + ERβ; as described detailed on Fig. 24. The results are expressed nanograms per milliliter (ng/mL) with mean and standard error of the mean. *p < 0.05; **p < 0.01; ***p < 0.001, using the paired ANOVA test or Friedman according the data normality.

Crohn's disease patients express the receptors ERα and ERβ in colon biopsies differently, comparing to healthy subjects, in which ERα are increased and ERβ decreased.[161, 162] Besides that, lower circulating ERβ/ERα ratio is highly correlated with CD endoscopic activity.[161, 163] Indeed, the specific activation of ERβ ameliorates experimental colitis DSS-induced by increasing T regulatory cells differentiation.[161, 164] On the contrary, ERα promotes T cell $CD4^+$ activation and proliferation contributing to colitis development.[165]

### *5.3 DHEA supplementation restrain intestinal inflammation*

To confirm our finds, it was induced experimentally colitis by dextran sulfate sodium (DSS) 2.5% in drinking water *ad libitum* for ten days in 15 male mice C57BL/6. To the treatment of the animals, DHEA (40 mg/kg/day) was emulsified in nanoparticles to a better drug delivery. Thus, the mice were subdivided in three groups: 1º - DSS without treatment; 2º - DSS plus empty nanoparticles; 3º - DSS plus DHEA emulsified in nanoparticles. The treatment was given orally every day.

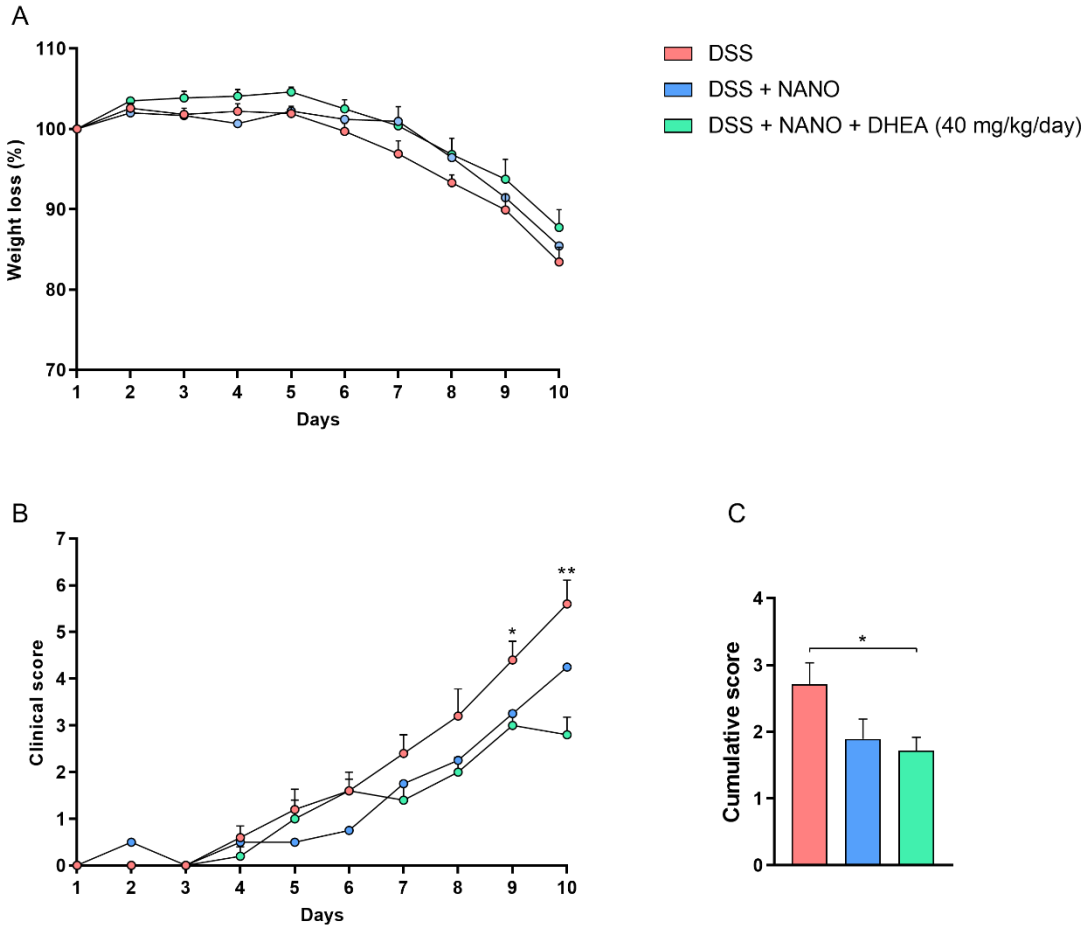


**Figure 27. DHEA has potential to treat experimentally colitis. (A)** Weight loss in percentage; **(B)** Clinical score, "*" means DSS vs. DSS+NANO+DHEA; **(C)** cumulative score from the day 4th. The results are expressed in mean and standard error of the mean. *p < 0.05, using the Kruskal-Wallis test.

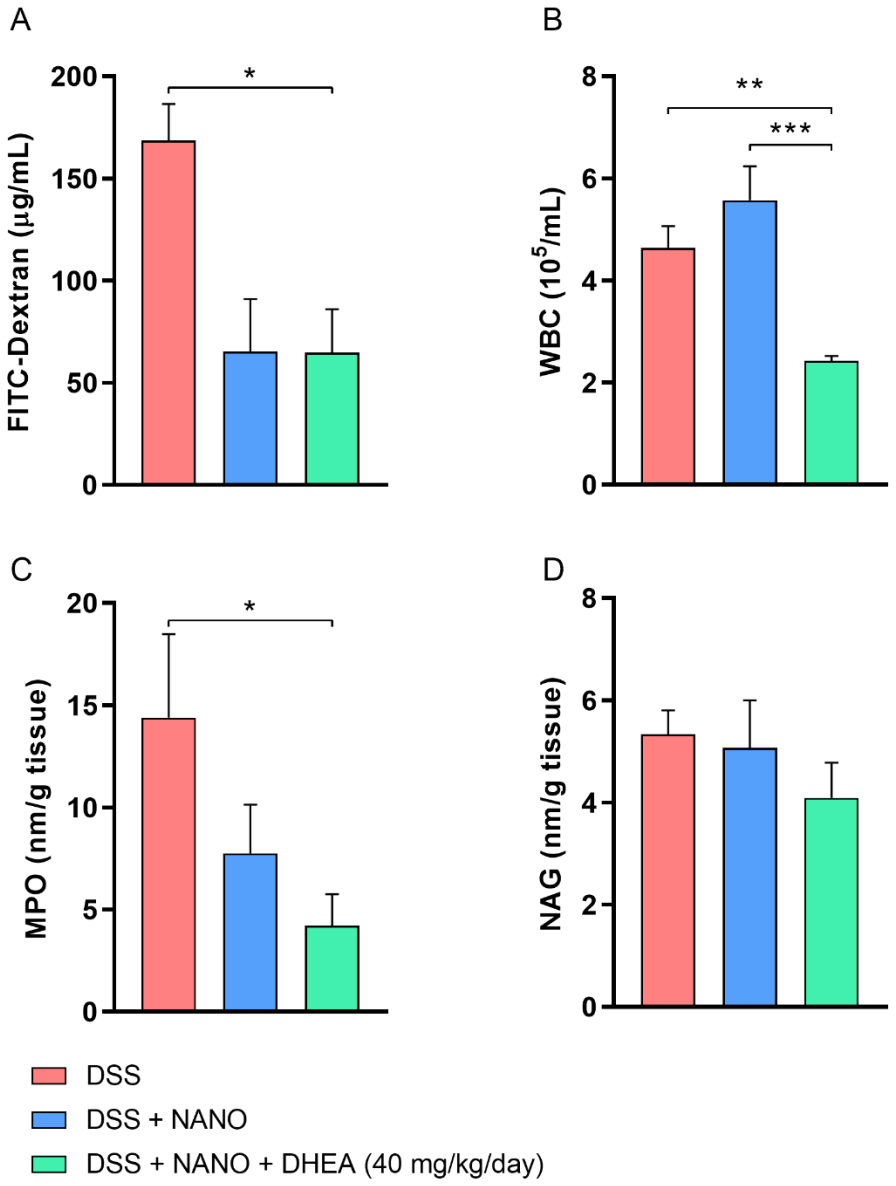


**Figure 28. DHEA reestablish mucosal barrier. (A)** FITC-Dextran in microgram per milliliter; **(B)** total white blood cells counting; **(C)** Myeloperoxidase (MPO) assay; **(D)** N-acetylglutamate synthase assay. The results are expressed in mean and standard error of the mean. $p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, using the Kruskal-Wallis test.

Although, no differences on weight loss (Fig. 27A), the treatment with DHEA significantly diminished the clinical score observed in the mice (Fig. 27B and 27C). Also, the DHEA *in vivo* supplementation improved the intestinal permeability (Fig. 28A) and decreased inflammatory markers such as, circulating white blood cells (Fig. 28B) and the neutrophil accumulation in the intestinal mucosa (Fig. 28C). Interestingly, the empty nanoparticle showed some protective effect as well.

DHEAS serum level is reduced in CD and inversely correlated to circulating IL-6 and TNF.[154, 155] The negative correlation between IL-6 and DHEA is also observed in healthy individuals.[166] Suggesting that DHEA may have an impact on IL-6 release and, in consequence, immune response. In fact, it has already been shown that DHEA supplementation regulates intestinal inflammation in experimental colitis DSS-induced by balancing Th1/Th2/Th17 responses.[160] DHEA also regulates the innate immunity in mice colitis by decreasing MAPK and NF-κB signaling pathways and impairing IL-1β production via NLRP3 inflammasome activation.[158, 159]

# 6 Conclusion

## 6. CONCLUSION

There is an unbalanced immunoendocrine interaction in Crohn's disease (CD) that is related to the disease outcome, specially concerning the sex steroid hormones. The expression of steroidogenic enzymes in colon of patients differs from healthy subjects, prioritizing the synthesis and activation of sex hormones. These differences are even more evident in non-responder patients to anti-TNF therapy, in which the genes expressed downstream to AR and ERα activation is the opposite that happens in heathy controls.

The hormone circulant levels of dehydroepiandrosterone (DHEA) and testosterone were reduced in CD. DHEA supplementation is able to dampers *in vitro* inflammation mediators' production. The treatment with DHEA *in vivo* restrains intestinal inflammation and ameliorates experimental colitis DSS-induced.

DHEA may be able to restore the unbalanced immunoendocrine interaction observed in CD and be a potential supporting therapy to inflammatory bowel diseases.

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**APPENDIX A**

**UNIVERSIDADE DE SÃO PAULO**
**Faculdade de Ciências Farmacêuticas de Ribeirão Preto**
*Comitê de Ética em Pesquisa*

Of. CEP/FCFRP nº. 048/2017
kms

Ribeirão Preto, 10 de outubro de 2017.

Ao Pós- graduando
**Murillo Duarte Silva**
Orientadora: Profª. Drª. Cristina Ribeiro de Barros Cardoso
FCFRP/USP

Prezado Pesquisador,

Informamos que o projeto de pesquisa intitulado "**EFEITO IMUNOMODULADOR DE DEHIDROEPIANDROSTERONA (DHEA) NA RESPOSTA IMUNE DE PACIENTES COM DOENÇA DE CROHN NÃO RESPONSIVOS À TERAPIA COM ANTI-TNF**", apresentado por Vossa Senhoria a este Comitê, Protocolo **CEP/FCFRP nº. 436 – CAAE nº 65063917.8.0000.5403,** foi aprovado *ad referendum* do Comitê de Ética em Pesquisa da FCFRP/USP em 07/07/2017, conforme Parecer Consubstanciado do CEP nº 2.162.611, sendo referendado na 170º reunião ordinária, realizada em 05/09/2017.

Lembramos que, de acordo com a Resolução 466/2012, item IV.5, letra d, o TCLE deverá "ser elaborado em duas vias, rubricadas em todas as suas páginas e assinadas, ao seu término, pelo convidado a participar da pesquisa, ou por seu representante legal, assim como pelo pesquisador responsável, ou pela(s) pessoa(s) por ele delegada(s), devendo as páginas de assinaturas estar na mesma folha. Em ambas as vias deverão constar o endereço e contato telefônico ou outro, dos responsáveis pela pesquisa e do CEP local".

Informamos que deverá ser encaminhado ao CEP o relatório final da pesquisa em formulário próprio deste Comitê, bem como comunicada qualquer alteração, intercorrência ou interrupção do mesmo, tais como eventos adversos e eventuais modificações no protocolo ou nos membros da equipe, através da interposição de emenda na Plataforma Brasil.

Atenciosamente,

PROFª. DRª. CLENI MARA MARZOCCHI MACHADO
Coordenadora do CEP/FCFRP

Comitê de Ética em Pesquisa FCFRP/USP
Avenida do Café s/n° - Monte Alegre – CEP 14040-903 – Ribeirão Preto – SP
Fone: (16) 3315-4213 – Fax: (16) 3315-4892 - cep@fcfrp.usp.br

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA
DE RIBEIRÃO PRETO DA UNIVERSIDADE DE SÃO PAULO

FMRP-USP
RIBEIRÃO PRETO

Ribeirão Preto, 02 de outubro de 2017

**Projeto de pesquisa**: "EFEITO IMUNOMODULADOR DE DEHIDROEPIANDROSTERONA (DHEA) NA RESPOSTA IMUNE DE PACIENTES COM DOENÇA DE CROHN NÃO RESPONSIVOS À TERAPIA COM ANTI-TNF"

**Pesquisador responsável**: MURILLO DUARTE SILVA

**Instituição Proponente**: Faculdade de Ciências Farmacêuticas de Ribeirão Preto – USP

"O CEP do HC e da FMRP-USP concorda com o parecer ético emitido pelo CEP da Instituição Proponente, que cumpre as Resoluções Éticas Brasileiras, em especial a Resolução CNS 466/12. Diante disso, o HCFMRP-USP, como instituição co-participante do referido projeto de pesquisa, está ciente de suas co-responsabilidades e de seu compromisso no resguardo da segurança e bem-estar dos sujeitos desta pesquisa, dispondo de infra-estrutura necessária para a garantia de tal segurança e bem-estar".

*Ciente e de acordo*:

Drª Marcia Guimarães Villanova
Coordenadora do Comitê de Ética em Pesquisa - HCFMRP-USP

Prof. Dr. Eduardo Barbosa Coelho
Coordenador Técnico Científico da Unidade de Pesquisa Clínica – HCFMRP-USP

## APPENDIX B

**UNIVERSIDADE DE SÃO PAULO**

**Faculdade de Ciências Farmacêuticas de Ribeirão Preto**

*COMISSÃO DE ÉTICA NO USO DE ANIMAIS*

# AUTORIZAÇÃO

Certificamos que a proposta intitulada "Papel da dehidroepiandrosterona (DHEA) nanoemulsionada na modulação da resposta imune intestinal em modelo de colite experimental", registrada sob nº 19.1.1154.60.5 sob a responsabilidade de Murillo Duarte Silva e Cristina Ribeiro de Barros Cardoso, que envolve a manutenção e utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto º 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi aprovada *ad referendum* em 15/07/2020 pela Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas de Ribeirão Preto (CEUA FCFRP).

Lembramos da obrigatoriedade de apresentação do relatório de atividades, em modelo da CEUA, para emissão do certificado, como disposto nas Resoluções Normativas do CONCEA.

Colaboradores: Camila Figueiredo Pinzan e Gilda Alves Carvalho Gatto.

| | |
|---|---|
| Finalidade | (  ) Ensino ( x ) Pesquisa Científica |
| Vigência da Autorização | 17/07/2020 a 17/05/2022 |
| Espécie/Linhagem/Raça | Camundongo isogênico C57BL/6 |
| Nº de animais | 232 |
| Peso/Idade | 20g/ 4-6 sem |
| Sexo | Macho |
| Origem | Biotério Central da PUSP-RP |

Ribeirão Preto, 15 de julho de 2020.

**Ana Patrícia Yatsuda Natsui**
Coordenadora da CEUA-FCFRP

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