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ORIGINAL ARTICLE

Gene Expression of *ABHD6*, a Key Factor in the Endocannabinoid System, Can Be Modulated by Female Hormones in Human Immune Cells

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Abstract

One of the main risk factors for the development of an autoimmune disease is to be a woman. Much attention has been given to the involvement of female hormones in their etiology and sexual bias, although the mechanisms behind this potentially strong contribution in disease susceptibility are poorly understood. *ABHD6* gene was recently identified as a risk factor for system lupus erythematosus and the risk was correlated with overexpression of the gene. ABHD6 is an enzyme that degrades the 2-arachidonoylglycerol, an endocannabinoid with immunomodulatory effects. Thus its degradation could contribute to immune dysregulation and development of autoimmune reactions. Sex hormones, such as estrogens, are believed to regulate important genes in the endocannabinoid pathway. However, no study was available regarding the effect of these hormones in human immune cells. In this study, *ABHD6* expression was evaluated by quantitative PCR in leukocytes from healthy male and females and in the presence of estrogen or progesterone (PG). A statistical increase in *ABHD6* expression could be detected in women. In the presence of estrogen or PG, a statistical upregulation of *ABHD6* was observed, and in a sex-dependent manner, as only female cells responded to stimulation. Our results suggest that female hormones can promote the overexpression of *ABHD6* in immune cells. This can potentially contribute to a pro-inflammatory scenario and partially explain the association of this gene in the development of LES, a highly female-biased disease.

Keywords *ABHD6* · Endocannabinoid system · Sex hormones · Autoimmune disease · Gene expression

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Introduction

The endocannabinoid signaling system is a group of specialized lipids, their receptors, and the enzymes that produce and degrade them. Through direct and indirect actions, endocannabinoids are known to modulate and influence a variety of physiological systems. In mammals it includes inflammatory processes, neuronal functions, memory, perception, and locomotor activity. Moreover, is involved in the etiology of certain human diseases, such as Crohn's disease and atherosclerosis, which reflects their eclectic endogenous signaling, influencing multiple metabolic pathways (Fine and Rosenfeld 2013). Interestingly, this system is probably very evolutionarily conserved as it is found from coelenterates to humans, and exists as an ancient plant signaling system regulating immune-related genes in response to infection and stress (Chapman 2000). Recent studies in animals indicate that the physiological effects of endocannabinoids can have important immunomodulatory effects and the cannabinoid system is believed to play an important role in immune homeostasis. In fact, immune cells express cannabinoid receptors, secrete endocannabinoids and have functional cannabinoid transport and breakdown mechanisms (Maresz et al. 2007). Among the endocannabinoids, the 2-arachidonoylglycerol (2-AG) acts as agonists who may activate cannabinoid receptors types 1 and type 2 (CB1R and CB2R) (Wen et al. 2015). Both CB receptor subtypes are expressed in neurons and in central and peripheral immune cells, and regulate degeneration and inflammation (Bernardi et al. 2007). The CB1R is expressed at high levels by most neurons and at a lower level by immune cell (Maresz et al. 2007). In contrast, the CB2R is highly expressed on the surface of several immune cells, such as the B and T lymphocytes, monocytes, natural killer (NK) cells, and neutrophils. Thus, the endocannabinoids are believed to play an important role in immune responses, and particularly in inflammation (Ueda et al. 2011; Katchan et al. 2016). The immunocannabinoid system is involved in immunosuppression of cell activation, modulation of T-helper cell types 1 and 2 (Th1 and Th2) and inhibition of pro-inflammatory cytokine production (Sipe et al. 2005). Thus, the 2-AG could be important to modulate innate and adaptive immune responses and the use of inhibitors of enzymes that breakdown the endocannabinoids are been investigated as a novel therapeutic modality against inflammatory and autoimmune diseases (ADs; Katchan et al. 2016)

One of those enzymes, α/β-hydrolase domain 6 (*ABHD6*), is a monoacylglycerol hydrolase enzyme that degrades the 2-AG directly at the site of its synthesis (Fisette et al. 2016). Thus, it can affect the 2-AG physiological levels and its action through the receptor CB2R, and, considering the immunomodulatory effect of 2-AG, potentially promoting the development of ADs (Sipe et al. 2005; Fisette et al. 2016). Indeed, a large mapping study revealed the genetic association of the 3p14.3 locus with systemic lupus erythematosus (SLE) in Europeans, and *ABHD6* was identified as the major susceptibility gene in this region (Oparina et al. 2014). Moreover, *ABHD6* expression was analyzed in lymphoblastoid cells, adipose cells and skin tissues and it was detected that the SNPs associated

with SLE correlated with expression levels of *ABHD6*, and only in lymphoblastoid cells, which may support a possible role of *ABHD6* in immune responses, including autoimmune-related pathways (Oparina et al. 2014).

There is great difficulty in identify the risk factors that contribute to the development of AD. Genetic susceptibility variants have been shown to have a modest influence on disease (Lettre and Rioux 2008). In that context, the major risk factor for an individual to develop several AD, such as autoimmune thyroid disease, Sjogren's disease, SLE, rheumatoid arthritis (RA) and scleroderma, is being a woman (Zandman-Goddard et al. 2007). During the reproductive age, the sex bias towards women reaches its peak, where the ratio can reach 10:1, depending on the disease (Schwartzman-Morris and Putterman 2012). This pattern indicates that factors associated with female gender/reproduction may have a strong influence on the development of AD. One aspect that has been increasingly considered is the influence of female sex hormones. Beside the fact that the gender bias is less significant before puberty and after menopause other evidences of this hormonal influence include variations in the activity of the disease during pregnancy and specific periods of the menstrual cycle, the fact that male individuals who undergo intensive treatment with estrogen for sex change has a prevalence of SLE very similar to women, as well as murine studies demonstrating that exogenous estrogens can trigger AD in susceptible strains (Rubtsov et al. 2010).

Sexual hormones play an important role in the function of both the innate and adaptive immune responses (Rubtsov et al. 2010). As well as playing a crucial role in sexual behavior and reproduction, it is believed that estradiol also has an important immunomodulatory role. This hormone influences the induction of cytokine production, maturation of B lymphocyte, autoantibody production, and lymphocyte T $CD4^+$ Th2-type responses (Pennell et al. 2012), although the mechanisms behind these effects are still unclear. Through the interaction with its specific nuclear receptors (ER), which act as transcription factors, estradiol can directly influence cell signaling and expression of numerous target genes, including several immune-related (Marino et al. 2006; Pennell et al. 2012). ER expression has been detected in several immune cells, such as B and T lymphocytes, monocytes, macrophages, neutrophils, dendritic cells and NK cells (Bouman et al. 2005; Pennell et al. 2012). However, the relationship between estradiol and the development of AD is complex. Estrogen probably has an etiological role that contributes to the triggering, severity and/or its exacerbation. Regarding the endocannabinoid system, there is evidence suggesting an influence of steroid sex hormones as estrogens can regulate important genes in the endocannabinoid pathway. This hormone stimulates the transcription of *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), a gene encoding an enzyme responsible for catalyzing the formation of *N*-acylethanolamines (NAEs), such as the major endocannabinoid *N*-arachidonoylethanolamine (AEA). Studies have revealed that E2 can directly stimulate the release of AEA from endothelial cells (Seki et al. 2007; Ayakannu et al. 2013). |Moreover, an increase of *ABHD6* mRNA levels upon stimulation with estradiol in uterine cells has also been observed (Boverhof et al. 2008; Oparina et al. 2014). No study was available regarding the effect of this hormone in human immune cells.

Progesterone (PG) is another female sex hormone that has immunomodulatory functions although the mechanisms behind this influence are less known. PG interacts with specific receptors, the PG receptors (PRs), which are found in both sexual and non-sexual cells, including immune cells (Tan et al. 2014). A few studies suggest that PG may act by suppressing the immune responses mediated by T-helper 1 (Th1) and Th17 and, in contrast, favor the secretion of cytokines by Th2 lymphocytes. Very few studies examined the effects of PG on the immune responses mediated by B lymphocyte. It is known that PG can suppress the differentiation pathway of these cells, but much more research is needed to clarify the impact of this hormone on this cellular group. In addition, this hormone may induce immune tolerance against fetal antigens during pregnancy (Hughes 2012). In human lymphocytes, PG has been shown to upregulate the activity of the fatty acid amide hydrolase gene and thereby decrease AEA plasma levels (Pauklin and Petersen-Mahrt 2009; Hughes 2012). In addition, has also been documented to downregulate uterine NAPE-PLD expression in mice, leading to a decrease in tissue AEA levels (Ayakannu et al. 2013). A possible relationship of this hormone on *ABHD6* expression or the endocannabinoid system in immune cells had never been investigated.

Here we investigated the impact of these hormones on expression of the gene *ABHD6* in male and female human immune cells and thus contributing to the understanding of the effect of female hormones in the endocannabinoid system as well as the etiologic mechanism involved in the gender bias in ADs.

Materials and Methods

Cell Culture and RNA Extraction

The samples group of the present study consists of 34 healthy individuals, being 11 men and 23 women. Buffy coats from healthy blood donors were collected at the University Hospital of Florianopolis (Hospital Universitário Polydoro Ernani de São Thiago). All women were adult premenopausal and both genders had similar mean age (28.1 ± 8.3 for women and 30.4 ± 7.2 for men). The study originally focused solely at women, the male sample group was added later for comparison and is therefore more limited in sample size. However, according to sample size calculation of two means two-sides, for the difference and standard deviation observed, power of 80% and p value of 0.05 the sample size should be at least 16 for women, and at least 10 for men.

A fraction of leucocytes was immediately collected in Trizol for RNA extraction at time point 0 h (ex vivo leucocytes). Another fraction of the cells was cultivated at 10^5/ml in RPMI culture without phenol-red, 2% charcoal-stripped fetal bovine serum, and 1% PEST at 37 °C and 5% CO_2. The cells were stored for a period of 12 h to acclimate, in order to eliminate the potential effect caused by previous exposure of the hormones of the individual. Cells were then treated with 100 nM 17b-estradiol (E2) or PG in two concentrations, 10 or 100 nM. E2 was used at this concentration according to our own previous standardization (unpublished data) and due to its extensive use in experimental stimulation

studies in vitro (Yahata et al. 2001; Nebel et al. 2010; Shen et al. 2010). PG on the other hand has been reported to cause a contrasting effect on immune-related pathways with the use of lower (10 nM) or higher (100 nM) concentrations (Hughes 2012). To the control group, the same amount of vehicle solution only was added. Cells were collected at the time 0, 12, and 36 h (stimulated and non-stimulated with different hormones and concentration) for RNA extraction. Cells were tested for viability with Trypan Blue at 12, 24, and 36 h. RNA extraction was carried out using standard Tri-Reagent® protocol according to manufacturer's instructions and RNA was quantified and qualified using nanovue (GE Healthcare Life Sciences) and gel electrophoresis. All cell culture reagents and hormones were purchased from Sigma Aldrich unless stated otherwise.

The study protocol was approved by the Institutional Committee of Ethics in Human Research (Number of the Institutional Approval Protocol 423.535, from 2015).

cDNA Synthesis and qPCR

500 ng of RNA was transcribed into cDNA through the high capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. Gene expression was performed using Fast SYBR® Green PCR master mix on an ABI HT7900 quantitative real-time PCR (qPCR). The following sense and anti-sense primers were used for amplification: *ABHD6*: F-TGAGGCTGGTCAGGAGTC AG and R-GACTTGCATGCCCAATGTCC. The pair of primers targets the two known splice variants of the gene (NM_001320126.1, NM_020676.6) as well as the predicted transcripts variants (XM_005265334.3, XM_005265335.3, XM_017006930.1) and generated an amplicon of 210 bp. PCR conditions consisted of initial denaturation at 95 °C for 20 s, followed by 40 cycles comprising 1 s at 95 °C and 20 s at 60 °C. All assays were performed in duplicates. All cDNA synthesis and qPCR reagents were purchased from Applied Biosystems. Expression was normalized to that of the gene coding for TATA-binding protein (*TBP*) (primers F-CACGAACCACGGCACTGATT and R-TCACAGCTCCCC AC CATGTT). $2^{-\Delta\Delta C_t}$ Calculations were performed, and gel of PCR products was performed and melting curves were analyzed at all qPCR measurements.

Statistics Analysis

Statistical differences in expression levels were calculated by Wilcoxon matching pairs two-tailed *t* test with GraphPad Prism software (available at http://www.graphpad.com). p values < 0.05 were considered significant between groups of samples. The charts display the difference in expression between the samples, measured by fluorescence that are converted to C_t values (cycle threshold), that relate to the original amount of mRNA for that gene in the sample.

ABHD6 Gene Sequence Analysis for Estrogen-Responsive Elements (EREs)

The sequence of the gene ±5 kb was analyzed using Ensembl (available at http://ensemble.org) for known ERE sequences, or described combination of half ERE elements with transcription factor binding sites based on the literature (Driscoll et al. 1998; Bourdeau et al. 2004).

Results

In the samples of RNA extracted immediately after collection, which denote the individual under normal conditions, it was possible to detect a difference in *ABHD6* expression between women and man in immune cells ($p = 0.021$; Fig. 1). After 12 h to acclimate, the expression of *ABHD6* by leukocytes was not significantly altered. When the immune cells were stimulated with estradiol, a discrete upregulation of *ABHD6* was observed, in a sex-dependent manner, since only female cells responded to E2 stimulation ($p = 0.046$; Fig. 2).

When the immune cells were stimulated with the lowest concentration of PG (10 nM), we observed a modest effect of *ABHD6* upregulation in female immune cells ($p = 0.032$). At higher concentrations (100 nM), this effect was more pronounced and the female cells displayed a significant positive regulation of *ABHD6* ($p = 0.024$).

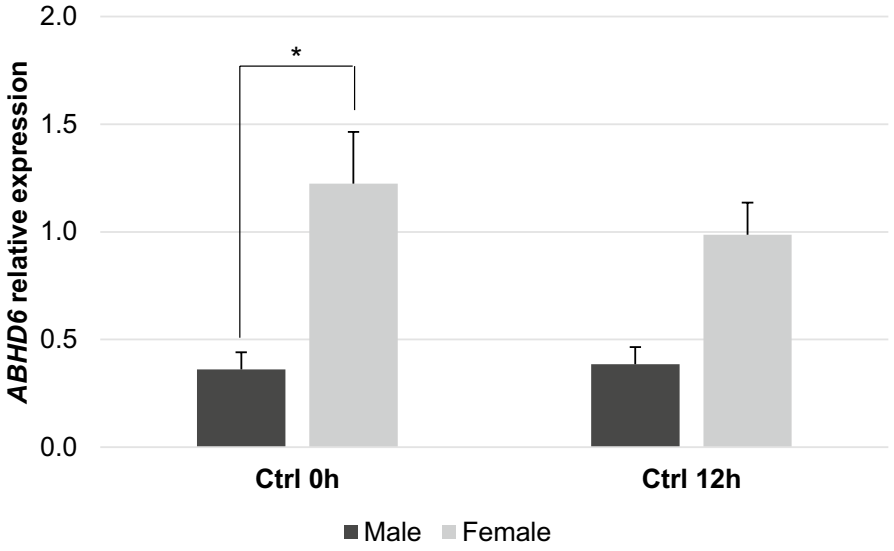

Fig. 1 Normalized mRNA expression levels of *ABHD6* in leukocytes from men (dark gray) and women (light gray) at the time of collection and after 12 h in culture with phenol-free RPMI and charcoal-stripped FBS. Fold difference in gene expression between groups is shown on the *Y* axis. Data represents mean values with standard error of means bars (SEM). $*p \leq 0.05$ were considered significant

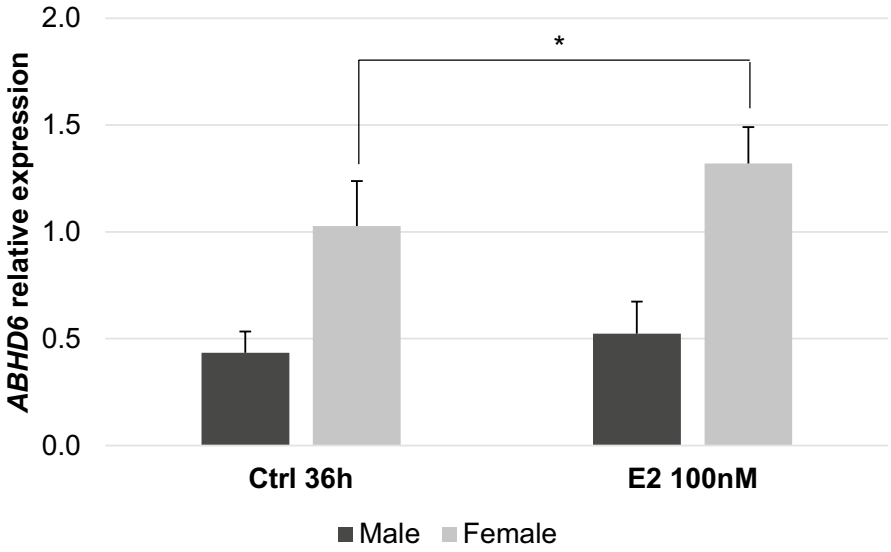

Fig. 2 Relative mRNA expression levels of *ABHD6* after 36 h (12-h acclimation + 24 h with or without estrogen stimulation) in leukocytes from men (dark gray) and women (light gray) in the control non-stimulated cell group and upon treatment with 100 nM estradiol (E2). Fold difference in gene expression between groups is shown on the *Y* axis. Data represents mean values with standard error of means bars (SEM). $*p \leq 0.05$ were considered significant, relative to the unstimulated control

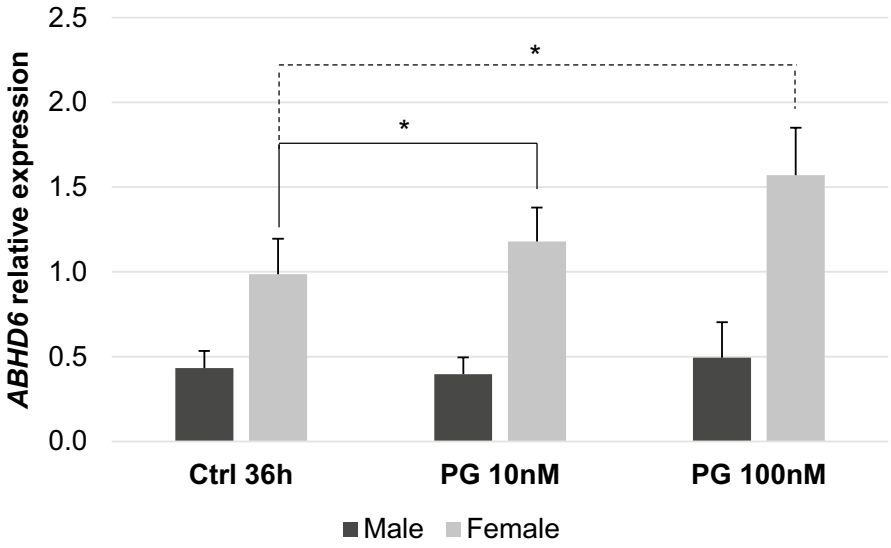

Fig. 3 Relative mRNA expression levels of *ABHD6* after 36 h (12-h acclimation + 24 h with or without estrogen stimulation) in leukocytes from men (dark gray) and women (light gray) in the control non-stimulated cell group and upon treatment with either 10 or 100 nM progesterone (PG). Fold difference in gene expression between groups is shown on the *Y* axis. Data represents mean values with standard error of means bars (SEM). $*p \leq 0.05$ were considered significant, relative to the unstimulated control

ABHD6 expression was not affected by either estrogen or PG, at any concentration, in male-originated immune cells (Figs. 2, 3).

Discussion

Our initial question was whether there was a difference in expression of *ABHD6* between men and women. Under normal conditions, female immune cells had a significantly higher expression than males. Interestingly, when treated with exogenous estrogen, a sex-dependent effect in *ABHD6* expression was observed. In female immune cells, there was a discrete but significant increase in *ABHD6* expression. In male cells however, there no difference in expression upon treatment.

In spite of the estradiol be considered primarily a female hormone that regulate reproduction and behavior in women, ER expression is present in different male tissues and in immune cells (Luetjens et al. 2006; Pierdominici et al. 2010). However, the difference in levels of expression of these receptors (Gillies and McArthur 2010) may explain the unequal responsiveness to estrogen of *ABHD6* between men and women.

Estrogen might influence gene expression by binding to its receptors (ligand-dependent transcription factors), and the interaction of the E2–ER complex with ERE sequences (EREs) located in the regulatory region of the target gene, recruiting coactivator proteins, acetylation of histones, remodeling of chromatin, and recruitment of transcriptional machinery, which affects the gene transcription (Driscoll et al. 1998; Bourdeau et al. 2004; Vrtačnik et al. 2014). To explain this induced *ABHD6* expression by estrogen, we searched in the promoter region of the gene for a ERE sequence. According to our analysis, no canonical or known ERE sequence is found in the promoter region or proximity of this gene. However, it has been revealed that numerous known target genes contain response elements that vary considerably from a traditional ERE sequence. The presence of half ERE sequence is able to bind to the ER and cooperate with cell signaling, although with a reduced affinity when compared to the canonical ERE sequence (Lone et al. 2004). In addition, ER activated by estradiol might also affect the expression of other genes by interacting and influencing the binding properties of other transcription factors, as seen with stimulating-protein-a (Sp1), nuclear factor-kB (NF-kB) and activator protein 1 (AP-1), resulting in even more complex variety (Lone et al. 2004; Asaba et al. 2015). Thus, the effect is independent of the presence of a classical ERE sequence and it allows to significantly increase the repertoire of genes regulated by estradiol (Driscoll et al. 1998; Vrtačnik et al. 2014).

A PG-induced upregulation of *ABHD6* in cells treated with 10 nM, and more pronounced with 100 nM could also be detected; and this effect was also restricted to female-originated cells. A similar effect has been observed in plasmacytoid dendritic cells exposed to PG. The treatment induced the expression of Interferon-α through the transcription of several genes with immunological relevance, and that effect was higher in plasmacytoid dendritic cells of healthy women than in men (Hughes 2012). Dendritic cells from murine females, when exposed to PG, proved to be more sensitive to the effects of PG when compared to male cells, and the effect was correlated

with inhibition of pro-inflammatory cytokine production (Butts et al. 2008). PG is a female sex hormone that also has receptors on peripheral blood immune cells (Luetjens et al. 2006). In males however, information regarding the expression of PRs are scarce and limited to reproductive tissues (Luetjens et al. 2006).

From the earliest descriptions of ADs, it has been observed that women are more frequently affected than men. Sexual dimorphism is observed in Sjogren's syndrome, autoimmune thyroid disease, scleroderma, multiple sclerosis, SLE, RA, and other AD (Whitacre 2001). And in addition to the difference in prevalence, differences in the disease manifestations are also observed (Whitacre 2001). These differences may be a consequence of the individual genetic predisposition, which interacts with the hormonal effect of estradiol and/or PG (Lockshin 2006). In this context, the role of sex hormones in modulation of several immune responses and triggering ADs has become increasingly discussed (Zandman-Goddard et al. 2007; Hughes 2012). Given its ability to activate directly or indirectly several genes including those of immunological relevance and that play an important role in autoimmune processes, although it is not known through which mechanisms, it is interesting to consider the effect on the endocannabinoid system found in this study.

Our results indicate that estradiol and PG may increase the expression of *ABHD6*. ABHD6, in turn, is known to degrate 2-AG directly at the site of its synthesis, ultimately decreasing 2-AG levels and the activation of its receptors (Fisette et al. 2016). The 2-AG has been extensively studied as an endogenous ligand of cannabinoid receptors in brain and other tissues, and its degradation reduces the activation of the cannabinoid system (Ueda et al. 2011). The immunosuppressive effect of cannabinoids has also been demonstrated in animal models with chronic inflammation. The use of synthetic cannabinoids was able to suppress IL-6, reducing the lesion of the articular tissue of these animals (Zurier et al. 1998). Synthetic cannabinoids can also decrease IL-6 secretion by human macrophages in vitro, suggesting that this compound may have a value for treatment of joint inflammation in patients with SLE, RA and osteoarthritis (Nagarkatti et al. 2009). It has already been shown that cannabinoids improve clinical disease in patients with multiple sclerosis (Maresz et al. 2007). Another study demonstrated that ABHD6 inhibition had anti-inflammatory and neuroprotective effects in a mouse model of traumatic brain injury and autoimmune encephalomyelitis (Wen et al. 2015).

With these information's combined it is plausible to suggest that ABHD6 can provoke pro-inflammatory effects that may contribute to the development of AD, and describes a direct correlation between sex hormones, ABHD6 expression and modulation of autoimmune-related responses.

In that line, it is also interesting to consider this correlation in the context of estrogen and/or PG-based oral contraceptives use and hormonal replacement therapy in AD patients. The safety of these has been established in a general manner but individual responses and the biological contexts are certainly determining factors defining its suitability.

Our study contributes to the understanding of AD etiology as well as the gender bias and support the model that female sex hormones modulate the immune system, possibly including the ABHD6 pathway, to increase risk of AD in genetically susceptible individuals. Our result might also contribute to the discussion

of the importance and possible biological consequences of the use of exogenous estrogen and PG, especially in AD patients.

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Compliance with Ethical Standards

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