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Sex-specific Variation in Gene Expression Patterns in Endotoxin-stimulated and unstimulated Human Leukocytes in Response to Exhaustive Exercise

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Running head: Sex-specific changes in gene expression and Exercise

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Abstract

The microarray data set of a recent study on mRNA changes induced through exercise (half-marathon; pre exercise, 30min post-Ex., 3h post-Ex., 24h post-Ex.; women in luteal phase *vs* men; 1h whole blood culture ± LPS) was reanalyzed with a special focus on sex differences. Inflammation related pathways TLRs, cytosolic DNA sensing and RIG-I like receptors were differentially activated between sexes in LPS-stimulated cultures. Individual evaluation of genes like TNIP-1, TNIP-3, IL-6, HIVEP1, CXCL3, CCR3, IL-8 and CD69 including the DNA methylation related gene DNMT1 confirmed a bias towards less anti-inflammatory regulation in women compared to men. A bias towards higher fat utilization and higher expression of antithrombotic genes MRVI1 and PLAU in males was also found. Several genes related to brain function (OLIG2, TMEM106B, DDIT4, and KMO) were also differentially activated between sexes. Some of these, like KMO in women and DDIT4 in both sexes, may potentially constitute neuroprotective mechanisms.

Key words: Exercise, sex differences, inflammatory genes, LPS stimulation, Toll-like receptors

Introduction

Despite the well-known fact that there are sex-specific physiological responses to exercise stress, the majority of exercise studies have been done in males. In general, women reveal lower concentrations of creatine kinase, an indicator of muscle damage after one hour of endurance exercise (63). Additionally, there is a reduced response of catecholamines after endurance exercise in women (29, 71). A lower utilization of carbohydrates by muscle and an increased use of peripherally mobilized fatty acids in women has also been demonstrated (24, 44). In addition to their obvious influence on our phenotype, gender differences are also relevant in the immune response. Overall, the activated NK cell and cytotoxic T cell response is higher in female as compared to male athletes. In addition, women in the second phase of their menstrual cycle revealed less anti-inflammatory response to exhaustive exercise as compared to men (1). The reason

for the differences between sexes in immune response to exercise seems to be largely related to fundamental differences in the immune systems of males and females. Generally, while men are more prone to bacterial, viral and parasitic infection and sepsis, women have a higher prevalence of a number of autoimmune diseases, including rheumatoid arthritis (RA) and multiple sclerosis (see Pennell et al., 2012). These fundamental differences between the immune systems of males and females suggest that gonadal hormones and genes of the X chromosome (approximately 1000 genes) may both contribute to this difference (22). While in general the impact of exercise on immune system functions has received considerable and increasing attention in recent years, it is still unclear to what extent gender and fluctuations in sex hormones during menstrual cycle influence immunological responses to exercise.

Generally, it is known that changes in estrogen levels are associated with changes in T helper lymphocyte (Th)1 and Th2 responses in a biphasic manner during the menstrual cycle, with Th1 responses heightened during menstruation and the luteal phase when estrogen levels are low, and with Th2 responses heightened during the follicular phase when estrogen levels are elevated (52). Numbers of circulating T-regulatory cells (Treg) also fluctuate with the menstrual cycle tending to be higher in the follicular phase (when estrogen levels are high) and lower in the luteal phase (8).

In the context of endurance exercise less is known about the sex- and menstrual phase-specific differences of the immune response. Recently, a microarray study by our group (49) summarized new data on sex- and menstrual phase- dependent differences in immunological responses to exercise. This study clearly demonstrated that the expression of pro-inflammatory genes was significantly up-regulated in women`s PBMCs in the luteal phase of their menstrual cycle as compared to the same women in follicular phase and men. Conversely, women in the luteal phase showed a strong trend towards down regulation of anti-inflammatory genes (49). Timmons and colleagues (2005) also found higher numbers of circulating neutrophils, monocytes and lymphocytes during the luteal phase than during the follicular phase in women using oral contraceptives (64). However, most studies reported no differences in cell counts and functions (11, 42, 45–47, 67), plasma cytokine levels (42, 66), and lymphocyte apoptosis (48) between the sexes. Most

studies did not control for the menstrual cycle and use of contraceptives at the time of testing.

To date, no microarray study has been performed on peripheral blood focusing on gender differences in response to exercise, particularly prolonged exhaustive exercise. We performed a detailed analysis of the microarray data set from our recent study (competitive half-marathon; men vs women in luteal phase; ex vivo culture of whole blood cells ± LPS stimulation before, 30min post exercise, 3h post exercise and 24h post exercise) using algorithms focusing on sex differences. Studying sex differences in response to exercise and in relation to pathogen contact is expected to yield new insights for better understanding sex-specific adaptations to exercise for athletic performance and health.

Methods and Materials

It should be noted that the results presented in this study share the same data basis with the results published recently by our group (2). In other words, the methodology of the present study including subjects, blood sampling, stimulation and incubation, RNA extraction and microarray analysis are the same as in our previous publication and have been described in detail (2).

Although, in that study, some sex specific results were mentioned, there was no systematic data analysis with focus on sex differences. In this study we report the results of running different algorithms, focusing on sex-specific regulation of gene expression on the existing set of microarray data.

In brief, eight (4 male and 4 female) well-trained athletes participated in an official half-marathon running. K3-EDTA blood samples were taken before and at several time points (30min, 3h, and 24h) after exercise. Whole blood was cultured with or without lipopolysaccharide (final concentration 10 ng/ml) for 1h and PaxGene Blood RNA kit was used to extract total RNA. Human Genome U219 Gene Chip-arrays (Affymetrix) were performed and microarray hybridizations were analyzed on the software platform R 2.12.0 with Bioconductor 2.10.0 (for more detail see publication Abbasi et al., 2014).

Cluster analysis for selected probesets was performed in R 2.15.1. Signal intensities were scaled and centered and the distance between two expression profiles was calculated using euclidian distance measure. Hierachical cluster analysis was performed with average linkage. Heatmaps were generated with Bioconductor package *geneplotter*.

Gene Ontology and KEGG Pathway analysis

In the lists of genes that were significantly differentially expressed with exercise and gender in our study, we conducted functional enrichment testing including Gene Ontology (GO) (9) and Kyoto Encyclopaedia of Genes and Genomes (KEGG; www.genome.jp/kegg/) pathways (33) to determine the relative enrichment of genes with common or related functionalities to gain insight into biological processes mediated by both exercise and gender. This might give an overview which biological and molecular processes are responsible for the observed changes in transcription. A one-sided conditional hypergeometric test was used to analyze the lists of differentially regulated transcripts for over-representation of GO categories in the two GO main branches "biological process" and "molecular function". GO categories with a p-value of less than 0.01 were called significantly enriched. In the same way, the lists were analyzed for over-representation of known signal transduction and metabolic pathways from the KEGG data base.

Results

Anthropometric and Exercise data

The Anthropometric data has been presented in our previous publication (2) with all runners successfully completing the half-marathon race (21.1 km) in an average running time of 95.5 ± 8 min (86-116min) for men and 114 ± 12min (96-129min) for women.

Hormonal status

Basal hormone concentrations of plasma estrogen, progesterone, LH, and FSH were measured to confirm the phase of menstrual cycle of the female subjects. The measurements confirmed that all female athletes were in luteal phase of their menstrual cycle (table 1).

Blood cell count in response to exercise

As described before, half-marathon running significantly increased total leukocyte counts for 3h post exercise ($P < 0.0001$) in both male and female athletes, with no sex-specific differences in cell count. Exhaustive exercise significantly increased the number and percentage of neutrophils at 30min- and 3h post exercise. The percentage of monocytes was decreased following exercise and remained attenuated for 3h post competition. The strong reduction in the percentage of lymphocytes also remained decreased for at least 3h post-exercise, but reached pre-exercise levels at 24h post-exercise.

Gene transcriptional and pathway regulation induced by exercise and differentially affected by gender (Time x Gender interaction)

Here, the changes in gene expression (up- or down regulation) from pre-exercise (t0) to post-exercise (t1,t2,t3) in male athletes in relation to the same status in female athletes are described (men post Ex. – men pre Ex.) — (women post Ex. – women pre Ex.). It should be noted that following this Time × gender algorithm (t × g alg.) no regulation results when up- or down-regulation through exercise occurs in male and female athletes in parallel. Regulation signifies that male and female athletes were affected by exercise in different ways. Thus, up regulation of this t × t alg. can mean that these genes were up-regulated in male athletes through exercise with no change in females, or that these genes were down-regulated in female athletes through exercise with no changes in males, or a mixture of both alterations. Vice versa, down regulation of t × g alg. indicates down-regulation of these genes in males or up-regulation of the same genes in female athletes in response to exercise.

Figure 1 shows the global transcriptional profiles in LPS-stimulated and un-stimulated whole blood cultures that differentially responded to exhaustive exercise between sexes (Figure 1).

In total, more genes were activated in men than women, but women had more activated genes at 24h post-exercise. In addition, LPS-stimulated cultures showed higher amounts of regulated transcripts compared to un-stimulated cultures for both sexes at any time point.

Table 2 shows the summary of most differentially and significantly regulated genes between sexes at each time-point (t × g alg.) in both cultures.

KEGG pathway analysis was used to assign the differentially regulated genes between sexes into functional categories in both LPS-stimulated and un-stimulated cultures. A detailed list of pathways which were significantly overrepresented in each group is shown in Table 3. KEGG pathways were activated for sex differences in LPS-stimulated only but not in un-stimulated cultures. The Toll-like receptor signaling pathway and Cytosolic DNA-sensing pathway were the only over-represented pathways for differentially regulated genes between sexes at 30min post-exercise. Four KEGG pathways were differentially over-represented for sex differences at 3h-post exercise and ``Epithelial cell signaling in Helicobacter pylori infection`` was the only significantly regulated pathway between sexes 24h after exhaustive exercise (Table 3).

Discussion

In the present study we used the microarray data from a previous study for a detailed analysis of sex-specific changes in gene expression profiling in LPS-stimulated and un-stimulated whole blood cultures following an exhaustive exercise. The rationale for using whole blood culture and the microarray technology has been discussed in detail in our previous publication (2). Fast and precise kinetics and avoidance of artefacts from in vitro manipulation characterize this strategy. There is widespread consensus in the scientific community that changes in gene expression in peripheral blood can be seen as mirror of changes induced by exercise in muscle or other organs.

Exhaustive exercise had significantly altered a row of genes in LPS-stimulated and un-stimulated blood cultures of male and female athletes. Exercise induced, in both male and female cultures, the differential expression of genes encoding products known to be involved in innate immune/inflammatory responses, metabolic responses, the cell cycle, apoptosis and regulation of transcription. In general, it had turned out that women showed a higher degree of pathway activation, while men showed higher numbers of activated genes at each time point after exercise. Up to now we have no stringent explanation for this finding. In another study, we had observed that women in their late luteal phase (like the ones investigated in this study) showed much higher numbers of

regulated genes immediately following 60min of moderate exercise compared with women in the follicular phase or men (49). Therefore, the two studies share at least the generally consistent finding that women in the luteal phase of their menstrual cycle show a different regulation following exercise as compared to men or women in their follicular phase.

Differential activation of signaling pathways between sexes

A prime finding of our present new specialized data analysis for sex differences is the observation that 5 differentially regulated KEGG pathways became apparent in endotoxin stimulated cultures (table 3). Of these, the TLR signaling pathway was clearly the most prominent with 7 differentially regulated genes 30min post exercise. Cytosolic DNA sensing and RIG-I like receptor signaling pathways followed. All three organize the primary reaction of the innate immune system to microbes of bacterial or viral origin. They are well interwoven and share expression of inflammatory cytokines and interferons as common endpoint. The two other pathways, which appeared in the late time points both concern infectious diseases and may be circumstantially co-reactive here. TLRs are external (e.g. TLR3,4) or internal (e.g. TLRs 3,7,8) receptors for bacterial cell wall components like LPS or microbial nucleic acids or analogues and induce synthesis of inflammation related cytokines (IL-1, IL-6, TNF-α) and /or type I interferons (IFN-β and IFN-α) (36). DNA sensing and RIG-I like receptor pathways are triggered mainly by virus derived nucleic acids and lead to the production of type I interferons, but also other inflammatory cytokines including activation of the necessary machinery for protein synthesis. Excessive activation of TLRs is implicated in the pathogenesis of infectious and inflammatory diseases (32, 41, 43). A recent work by Khan et al (2010) showed higher response by TLR-7 and TLR-8 but not TLR-4 and TLR-3 in healthy females as compared with males.

The differential pathway activation between sexes as proven by the above mentioned KEGG analysis, confirms that there are significant differences between the reaction of men vs women in luteal phase in the setting of exercise plus pathogen stimulation. It also showed that exercise does indeed interact with early steps of the pathogen response, involving at least 3 major pathways of innate immunity. In future, a comparison

including women in their follicular phase may be of interest. Also, the KEGG analysis does not provide any information on the nature of the observed differences. Some of these will be discussed below.

Exercise and the inflammatory response

Several studies have shown that exercise induces changes in pro-inflammatory, but also in prominent anti-inflammatory genes (2, 15, 18, 49, 57–60). About 40% of all genes changed through exercise in peripheral blood are related to inflammation (presented in ISEI symposium-Australia 2013). Previous investigations have demonstrated that in men and women in follicular phase, gene expression changes are quite comparable and show a strong anti-inflammatory bias, while this bias was far less prominent or absent in women in their luteal phase. The differences in (inflammation-related) pathway activation between men and women in luteal phase as pointed out above are nicely compatible with this picture even if data on women in follicular phase are missing. In the following, some genes will be considered in more detail to get more information on the nature of the observed pathway activation differences.

Within the TLR pathway, TNIP-1 and TNIP-3 (TNFAIP3 interacting protein-1, -3) are potent multiple action inhibitors (68). Their activation became only apparent in LPS-stimulated cultures, and men showed highly significant activation while women showed only a trend. Similarly, IL-6 and the tumorigenesis controlling gene HIVEP1(30) which are both clearly anti-inflammatory in their effects were significantly higher activated in men.

In contrast, several genes with prominent pro-inflammatory function such as IL-8, CXCL3, CCR3, and CD69 were also differentially regulated between sexes. While IL-8, a major player in innate immunity, did not change in male athletes, it was significantly up-regulated in unstimulated cultures of female athletes. Similarly, CXCL3, a strongly inflammatory chemokine, was significantly down-regulated in male athletes while significantly up-regulated in female athletes. Furthermore, mRNA of the chemokine receptor CCR3 was significantly down-regulated in male compared to female athletes. Contrasting this, LPS-stimulated (but not unstimulated) expression of CXCL10 mRNA was more strongly down-regulated in female compared to male athletes (6fold vs 4fold,

respectively). LPS-stimulated and unstimulated expression of CCL5 (RANTES) mRNA was also significantly down-regulated following exercise but with no differences between sexes. It should be noted that all chemokines mentioned above have potent pro-inflammatory activities in addition to their chemotactic properties and have been implicated in the progression of several inflammatory and autoimmune diseases (38, 40). CD69, a lymphocyte activation molecule, which is also involved in the pathogenesis of chronic inflammation (26), was also significantly down-regulated in both sexes with male having more pronounced regulation than female athletes. A significant down-regulation of CD69 mRNA after 60 min of post-exercise recovery had also been detected in response to an acute bout of 30min exercise (18). Still the behavior of this molecule has to be interpreted with caution, since we had a 2-4 fold reduction of lymphocyte percentage post exercise in this study which may be responsible for the observed down-regulation.

A further molecule, CD163, although not significantly different between sexes, may deserve a short discussion in this context. Expression of mRNA was activated through exercise in male and female athletes. Expression was slightly reduced in LPS-stimulated as compared to unstimulated cultures at all times, and in females there was a trend toward less up-regulation as compared to males. CD163 is a specific marker for M2 type macrophages and therefore likely to represent an exercise induced M1 to M2 switch. M2 macrophages are important in resolution of inflammation (13, 53) and can be induced by dexamethasone in monocytes (65), and can thus be interpreted as part of the anti-inflammatory effect of exercise. This confirms previous reports of M2 macrophages induction by acute and chronic exercise (10, 31, 35). We find it intriguing to see that induction of M2-specific mRNA (CD163) by exercise is a very fast process and that the due conversion of this process by LPS is obviously just beginning within this short time frame. In this context it may be interesting that, for this study, induction of ARG1, a functional marker of M2 macrophages, has been documented already (1). There was, however, no difference between sexes or stimulation modality in ARG1.

Taken together, in exercise induced gene regulation, we see a sex/menstrual phase specific bias towards less anti-inflammatory regulation in female athletes as compared to

male ones. KEGG pathway analysis revealed differences only in LPS-stimulated cultures, underlining the benefit of testing the interaction of exercise with pathogen contact.

Appearance of sex-specific differences in KEGG pathways of pathogen receptors TLRs, DNS sensing and RIG-I like receptors, highlights this message and has been shown here for the first time. Our results confirm previous findings from our group, but also from non-exercise related studies pointing to an inflammatory bias of women in their luteal phase (14, 39, 49, 70).

Sex-specific regulation of DNA methylation genes by exercise

In our recent report we had already presented that DNA methylation genes such as DNA methyltransferase-1 (DNMT1) and HemK methyltransferase family member 1 (HEMK1) were regulated following exercise. This was the first report of regulation of DNMT1 mRNA in peripheral blood. The present analysis shows that both genes are regulated in a sex-specific way. DNMT1 mRNA was strongly down-regulated by exercise in men (4 fold) with or without LPS stimulation, while women showed only mild regulation.

DNMT1 is classically referred to as a maintenance methyltransferase, although it also has de novo methylation capabilities (12). Several studies have recently referred to the role of DNMT1 in inflammation and inflammation-related disorders (19, 20, 23, 28). An interesting work by Dunn et al (2014) showed that disturbed blood flow (d-flow) controls epigenomic DNA methylation patterns in a DNMT-dependent manner, which in turn alters endothelial gene expression and induces inflammation. According to these findings DNMT1 inhibition in endothelial cells inhibited d-flow–induced inflammation in vitro (20). Therefore inhibition of DNMT1 in blood cells might represent an anti-inflammatory regulation and protective function for exercise, and the sex specific regulation observed in this analysis is well in line with the findings discussed in the previous chapter. Alteration in DNA methylation genes by sex hormones and menstrual cycle have been shown in brain and other tissues by several groups (37, 72). In light of the experiments by Dunn et al (see above) it can be speculated that DNMT1 expression in endothelium may be one of the target molecules mediating exercise dependent anti-inflammatory effects on the level of endothelium. We think that this hypothesis deserves further exploitation.

Sex differences in metabolic pathways

As expected, exhaustive exercise induced the expression of genes encoding metabolic proteins. Some of these genes were differentially expressed between sexes.

An interesting finding of the present study that probably links inflammation to metabolism is the induction of G0S2 (G0/G1 switch gene 2) gene in unstimulated culture of both sexes, with female having higher expression than male athletes. G0S2 is a potential inhibitor of ATGL (adipose triglyceride lipase) and lipolysis in adipocytes and fat explants. The expression of G0S2 is increased in response to insulin, glucose, and ligands for the PPAR family of transcription factors (73, 75). In contrast, TNF-α can drastically decrease the level of G0S2 mRNA, stimulating basal lipolysis in adipocytes, which may contribute to hyperlipidemia and peripheral insulin resistance (74). In addition, overexpression of G0S2 has been shown to decrease TNF-α-stimulated lipolysis mediated by overexpressed ATGL and CGI-58 (74). Therefore, G0S2 likely controls triacylglycerol (TAG) turnover in adipocytes and non-adipocyte cells, and alteration of its expression may be a way via which nutritional and hormonal factors regulate lipid homeostasis (74).

It may be suggested that the exercise-induced up-regulation of the G0S2 gene probably could ameliorate insulin resistance via inhibiting TNF-α- and ATGL-stimulated lipolysis and hyperlipidemia. Unfortunately there is no evidence regarding the sex-specific regulation of the G0S2 gene in human blood cells, particularly in response to exercise. The finding that female showed higher and more prolonged expression of G0S2 mRNA compared to male athletes in response to exercise may reflect less lipolysis in female compared to male. This is in contrast with the old notion that typically women have higher amounts of systemic lipolysis/FFA release and FFA oxidation as compared to men in response to exercise (7, 16, 27). However, these reports did not take into account menstrual cycle phase-specific effects.

We are aware that we cannot rely on single gene expression data to conclude about the sex-specific metabolism in response to exercise, but further gene data from the present study demonstrates also higher fatty acid utilization in male than female athletes. Exhaustive exercise significantly increased the expression of PDK4 (pyruvate dehydrogenase kinase, isozyme 4) gene in unstimulated culture of male athletes, while

females showed a slight down-regulation in the expression of PDK4 gene expression following exercise. PDK4 is a well-known isoform of PDH kinase (PDK) family that suppresses glucose oxidation by its inhibitory effect on the pyruvate dehydrogenase complex leading to an increase in fatty acid utilization. The rapid induction of PDK4 gene in male athletes was followed by a decrease towards resting values after 3h post exercise. It has been proposed that increased PDK-4 activity is contributing to the reduction of PDH activity and carbohydrate oxidation during and after prolonged exercise (Figure 2). To our knowledge, there is no evidence available regarding the regulation of PDK-4 mRNA in human peripheral blood cells in response to exercise, but findings from a muscle study showed a negative correlation between decreased PDH activity and increased PDK activity (69). The induction of PDK4 in the present study in male athletes, but not female, suggests that, in the setting of our study males used less carbohydrate than females, favoring fat utilization to feed the citrate cycle in males.

The induction of PDK4 gene in response to exercise in the present study is in accordance with the finding of Pilegaard et al (2004) who found a marked increase in PDK4 transcription and PDK4 mRNA in human skeletal muscle during prolonged exercise and after both short-term high-intensity and prolonged low-intensity exercise (54). Watt et al (2004) also reported a rapid induction of PDK-2 and PDK-4 activity in response to prolonged moderate-intensity exercise (69).

The change in PDK-4 gene expression could also be important from the view point of immunometabolism. Since activation of TNF-α and NF-κB by LPS could reduce the PDK-4 gene expression and therefore fatty acid oxidation in embryonic rat heart-derived H9c2 myotubes (51, 55), the induction of PDK-4 in blood cells in response to exercise might be due to the induction of PPARβ/δ and suppression of TNF-α and NF-κB signaling pathway. We have shown in our previous study that exercise significantly suppressed LPS-stimulated TNF-α in human blood cells (1).

Another finding that makes this hypothesis stronger was the long time up-regulation of PFKFB2 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2) gene in unstimulated cultures of female compared to the male athletes. The protein encoded by this gene is involved in both the synthesis and degradation of fructose-2,6- bisphosphate, a regulatory molecule that enhances generation of pyruvate from glucose. Therefore it can be

concluded that long-time activation of this gene in females compared to males suggests less bias in females toward fat utilization. The summary of metabolic gene regulation in response to exercise is shown in figure 2.

Exercise differentially interferes with hemostasis between sexes

Among the genes, which were differentially expressed between sexes in response the exercise, we found several genes that are involved in the regulation of homeostasis. LPS-stimulated expression of MRVI1 (murine retrovirus integration site 1 homolog) and PLAU (Plasminogen activator, urokinase) genes were among the top 10 most significantly regulated genes between sexes. MRVI1 (which is also called IRAG) inhibits platelet aggregation and activation, and PLAU is known for its potent antithrombotic activity, reflecting their important role in the regulation of homeostasis. For both genes, induction was more pronounced in male athletes, but female athletes had higher resting levels, suggesting that females may have naturally higher control on their homeostasis even during exercise, but males display a stronger response to exercise. This could be related to female sex hormones and their menstrual phase cycle. To date there is no evidence regarding the induction of MRVI1 gene in response to exercise, and this is the first study showing sex-specific change in MRVI1 gene expression following exhaustive exercise. Only one study has mentioned the induction of PLAU mRNA in the muscle of exercising people following an acute bout of exercise (17).

Exercise differentially modifies the expression of genes relevant for brain function and structure

There is now ample and accumulating evidence that exercise has beneficial effects on physiological and cognitive functions of the brain. Most of the studies have focused on central changes in neurotransmission, neurogenesis, growth factors, and blood flow and, hence, less attention has been given to peripheral factors that may affect brain function during exercise. In particular, possible links to cells and molecules of the immune system have not been investigated in depth.

Modification of OLIG2 and TMEM106B genes through exercise has already been mentioned in our first publication (2). The present analysis confirms that this regulation

was sex-specific (figure 3.A-B). OLIG2 (oligodendrocyte transcription factor 2), which plays an important role in oligodendrocyte differentiation and specification, was significantly down-regulated by exercise but only in LPS-stimulated cultures and only in male athletes. Females showed no change in the regulation of this gene. In contrast to OLIG2, TMEM106B was significantly (LPS) or mildly (no LPS) up-regulated in men while it was significantly (no LPS) or mildly (LPS) down-regulated in women. Over-expression of TMEM106B has been associated to dementia, namely frontotemporal lobar degeneration with TDP-43 pathology. While the sex-specific regulation of these genes is a new and potentially interesting observation, we need to confess that up to now we do not know how this may relate to peripheral and central nervous system function.

KMO (Kynurenine 3-monooxygenase) which was significantly down-regulated in LPS-stimulated cultures of female athletes may offer more easy access to possible interpretation (figure 3.C). KMO is a pivotal enzyme of the kynurenine pathway (KP) of tryptophan degradation and catalyzes the conversion of kynurenine to 3-hydroxykynurenine (3-HK). KMO and 3-HK, both can readily cross the blood– brain barrier and excess levels of these are associated with increased neuro-inflammation, and decreased neuroplasticity, and are implicated in the pathology of neurodegenerative and psychiatric diseases like depression (61). On the other hand, inhibition of KMO was shown to have opposite effects through formation of kynurenic acid (KYNA), which has potent neuroprotective properties (5, 25, 76). Inhibition of pathogen-induced KMO activation through exercise may therefore be interpreted as part or at least as mirror of a neuroprotective mechanism in women who are in an inflammation prone phase (luteal phase) and encounter inflammatory stimuli like LPS. This is the first report of KMO regulation through exercise. Exercise induced increases in kynurenic acid via KAT enzymes in muscle associated with brain protective functions, has been reported very recently (4).

DDIT4, an inhibitor of mTOR signaling, is another interesting molecule involved in synaptic loss, neural atrophy and depressive behavior (34, 50). Following slight non-significant induction at 30min post exercise, it was markedly downregulated 3h post exercise in unstimulated cultures in men significantly more than in women (figure 3.D). Inhibition of DDIT4 can also be interpreted as neuroprotective and antidepressive

regulation through exercise. The meanings of the slight but significant differences between sexes remain presently unclear.

Four further genes (VEGFA, IGF1R, IGF2R and FGD4) involved in peripheral and/or central nerves growth and in myelination (3, 6, 21, 62) were also strongly induced by exercise in LPS-stimulated and unstimulated cultures. Three of them (IGF1R, IGF2R and FGD4) showed significant but only minor differences between sexes with women having slightly less induction than men. The extent of the intersex differences in these genes is however so small that their biological meanings is questionable in our view.

Exercise and Spermatogenesis related genes

As depicted in figure 3 (E, F), two important spermatogenesis-related genes - besides their inevitable sex-specific difference in activation- showed significant regulation through exercise. Both were down-regulated at 30min and 3h post exercise. A negative impact of exercise on spermatogenesis has been shown earlier (56).

Conclusion

Taken together, the most prominent finding of this analysis is that there were sex-specific differences in activation of inflammation-related pathways TLRs, cytosolic DNA sensing and RIG-I like receptors. Individual considerations of inflammation related genes like TNIP-1, TNIP-3, IL-6, HIVEP1, CXCL3, CCR3, IL-8 and CD69 including the methylation related gene DNMT1 confirms that there was a bias toward less anti-inflammatory activation in the women tested who were all in the luteal phase of their menstrual cycle. Other findings were a bias toward higher fatty acid utilization and lower pyruvate generation in men as compared to our women. Further, men showed higher activation of antithrombotic genes MRVI1 and PLAU through exercise while women had a higher baseline. Finally, exercise also modified a set of genes related to brain function and structure (OLIG2, TMEM106B, DDIT4, and KMO), with significant (but in part only minor) differences between sexes. So far we find it difficult to present a reasonable interpretation for most of the observed differences in these brain related genes, except that we see hints for a neuroprotective background of regulation of KMO in women and DDIT4 in both sexes (Figure 4).

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Hierarchical cluster analysis of all transcripts which were significantly changed by exercise and gender (t × g alg.). Rows correspond to probeset ids, columns correspond to samples. Red: high expression, green: low expression.

Figure 2. Sex-specific regulation of metabolism in response to exercise. A bias toward higher fatty acid utilization and lower pyruvate generation was observed in men as compared to women.

Figure 3. Sex-specific regulation of genes relevant to brain function (A, B, C, D) and spermatogenesis (E, F) in LPS-stimulated and un-stimulated cultures in response to exhaustive exercise. X axis shows the time points with 0,0 corresponding to t1 (before Ex); 1,0 corresponding to t1 (30 min post-Ex); 2,0 corresponding to t2 (3 h post-Ex); 3,0 corresponding to t3 (24 h post-Ex). Y axis shows normalized signal intensities (log2).

Figure 4. Possible mechanisms for neuroprotective function of exercise and differences between sexes. DDIT4, a gene which is associated with promotion of neuroal atrophy and blocks mTOR signaling pathway to neural plasticity, was down-regulated through exercise, more so in men than in women (in luteal phase). Conversely, KMO, a pivotal enzyme of the kynurenine pathway of tryptophan metabolism, associated with induction of depression, was down-regulated in women only. Growth factors VEGFA, IGF1R, IGF2R were also induced by exercise with minor differences between sexes. Finally, FGD4 mRNA, a myelination inducing gene, was significantly enhanced by exercise in both sexes with men showing more pronounced regulation. Overall, the observed gene expression regulations may be interpretable as neuroprotective effects of exercise.

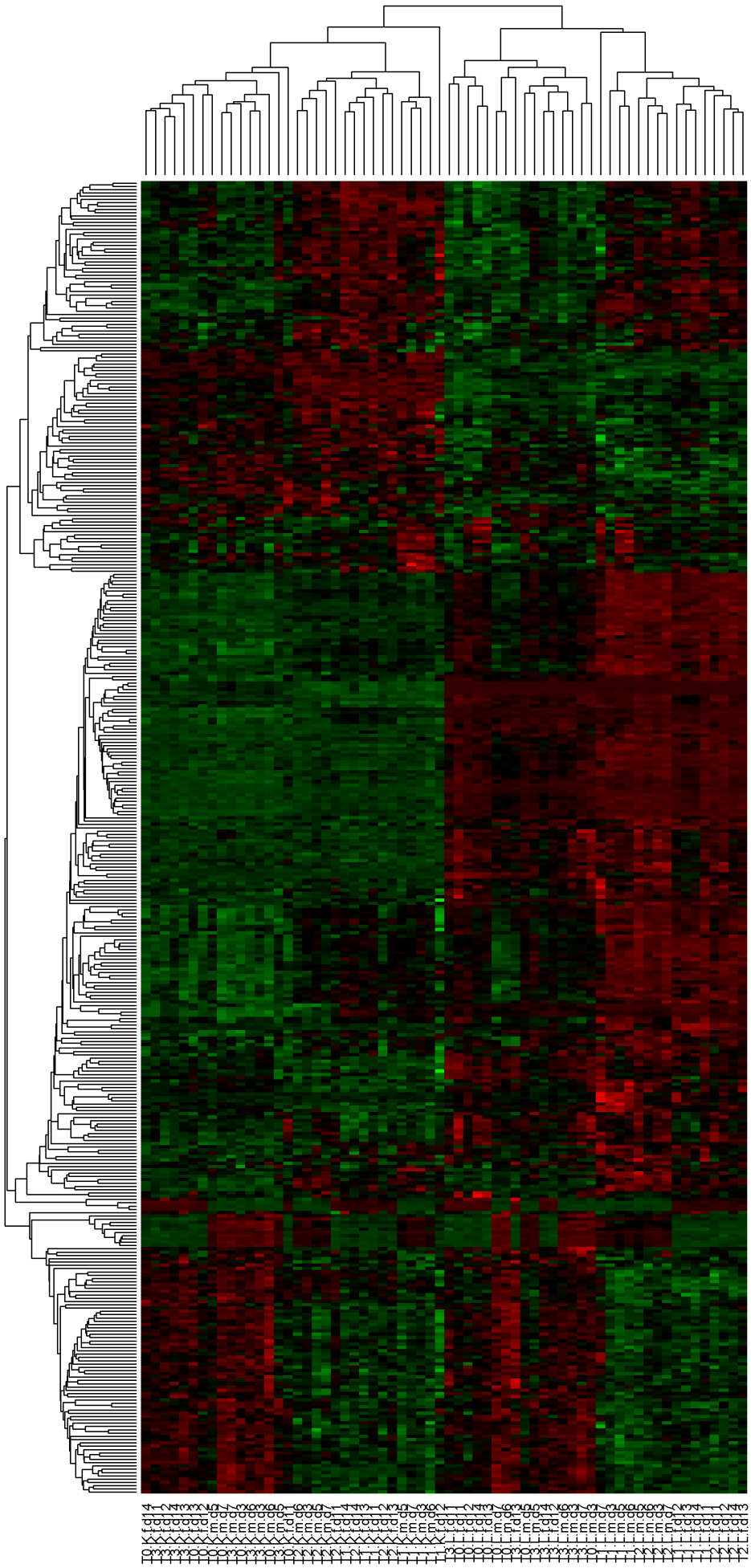
Table legends

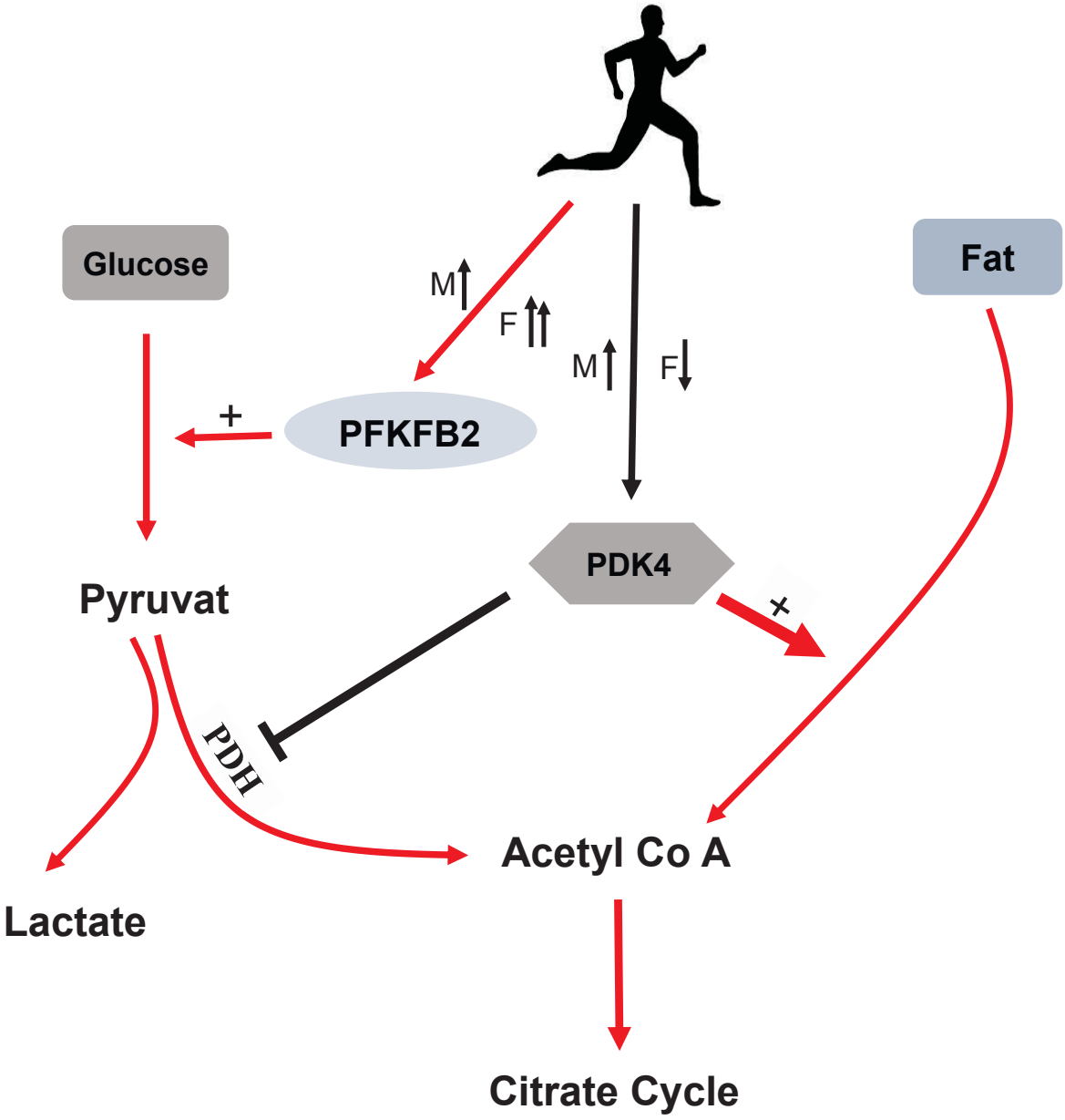
Table 1. Hormonal status of female athletes at baseline

Table 2. Most differentially regulated genes between sexes in each time-point (t × g alg.) in both cultures.

Table 3. The KEGG pathways significantly over-represented between male and female athletes in LPS-stimulated cultures following exhaustive exercise.

Time X Gender




Glucose
Fat
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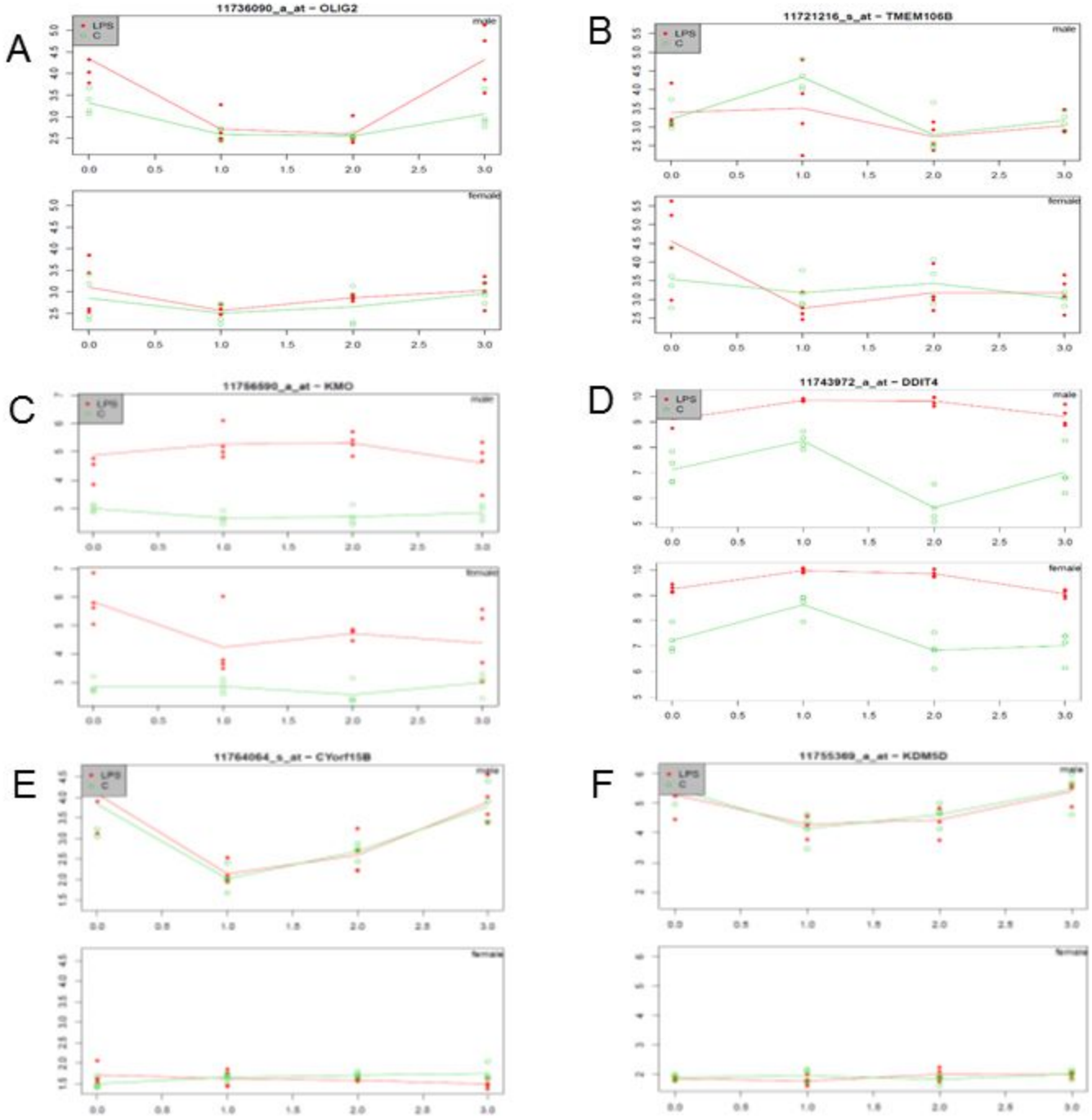
+
PFKFB2
PDK4
Pyruvat
PDH
Acetyl Co A
Lactate
Citrate Cycle


A
11736090_a_at – OLIG2
B
11721216_s_at – TMEM106B
C
11756590_a_at – KMO
D
11743972_a_at – DDIT4
E
11764064_s_at – C1orf15B
F
11755369_a_at – KDM5D
LPS
C
male
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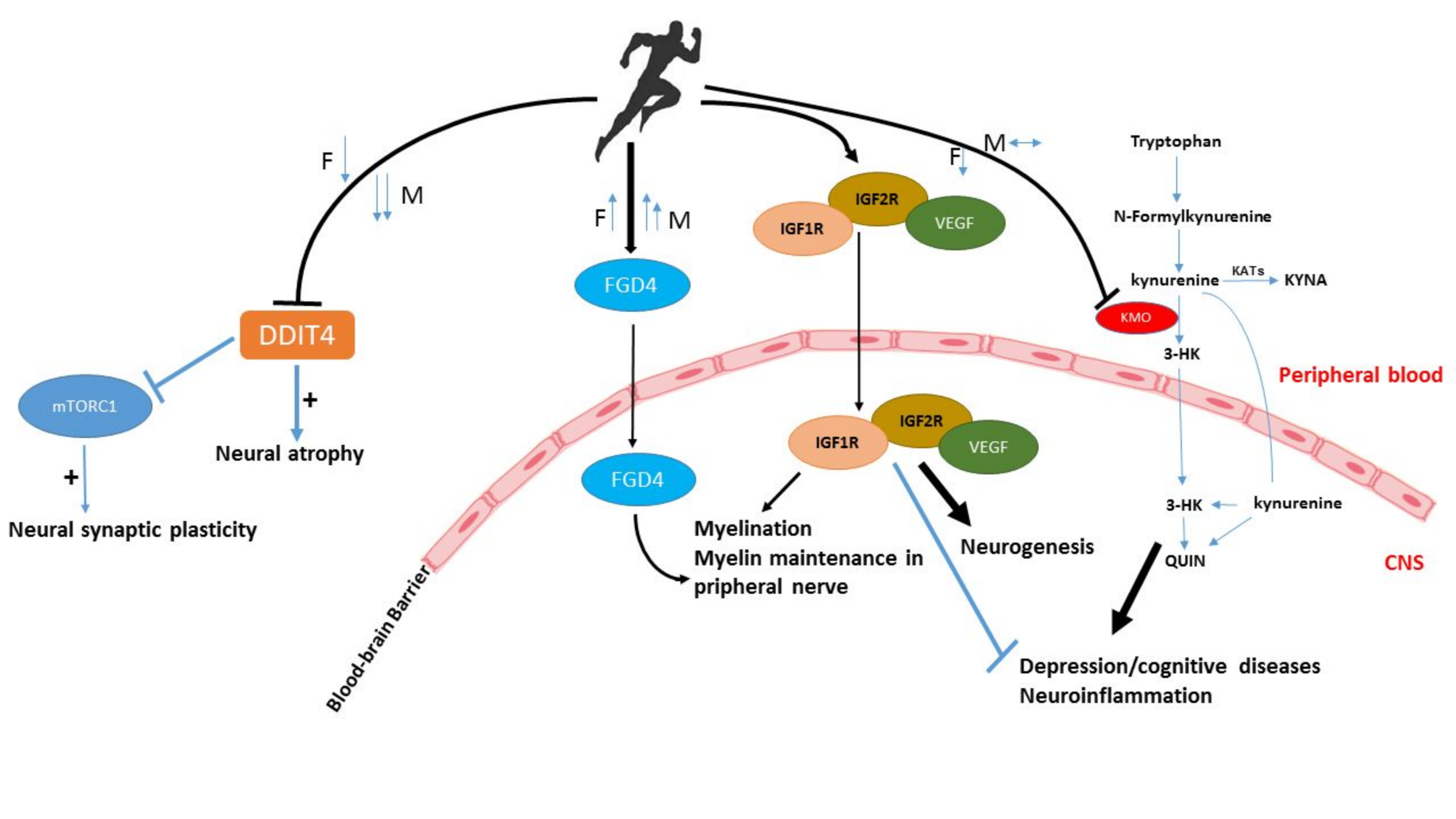
F
M
DDIT4
mTORC1
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Neural atrophy
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Neural synaptic plasticity
F
M
FGD4
FGD4
Myelination
Myelin maintenance in pripheral nerve
F
M
IGF2R
IGF1R
VEGF
IGF2R
IGF1R
VEGF
Neurogenesis
Depression/cognitive diseases
Neuroinflammation
Tryptophan
N-Formylkynurenine
kynurenine
KATs
KYNA
KMO
3-HK
3-HK
kynurenine
QUIN
Peripheral blood
CNS
Blood-brain Barrier

Table 1. Hormonal status of female athletes at baseline

LH (IU/l)	FSH (IU/l)	Estradiol (pmol/l)	Progesterone (pmol/l)
5.2 ± 4.07	5.1 ± 3.08	356.62 ± 339	4.48 ± 3,84

Values are in mean ± SD

Table 2. Most differentially regulated genes between sexes in each time-point (t × g alg.) in both cultures.

Gene Symbol	Log2 FC in Un-stimulated culture			Log2 FC in LPS-stimulated culture		
	T1	T2	T3	T1	T2	T3
MRVI1	1,967	0,428	0,424	1,917	0,866	1,298
PDK4	1,817	0,527	1,132	0,328	0,109	0,075
XIST	1,590	1,0798	-0,715	0,1970	0,277	-0,549
TMEM106B	1,477	-0,309	0,474	1,912	0,735	1,024
CYorf15B	-1,964	-1,345	-0,306	-1,848	-1,350	0,025
IL8	-1,795	-2,040	-0,863	-0,527	0,453	0,133
DNMT1	-1,481	-1,246	-0,369	0,478	-0,292	0,395
KDM5D	-1,405	-0,810	-0,131	-0,922	-0,988	-0,026
CCRL2	0,841	1,282	0,764	0,897	0,736	0,557
FAM129B	0,533	1,269	0,744	0,532	0,459	0,387
VASH1	0,567	1,053	0,843	0,428	0,072	0,191
LDLR	0,123	1,027	0,910	0,136	0,000	0,626
CCL4L1 /// CCL4L2	-1,334	-1,948	-0,062	-0,047	0,183	-0,015
CD69	-0,337	-1,436	-0,780	-0,346	-0,102	-0,144
CDKN1C	-0,754	0,253	1,168	-1,037	-0,917	0,202
DICER1	-0,043	0,058	1,042	-0,152	-0,165	0,214
TNIP3	-0,025	0,020	-0,028	3,371	1,228	0,582
OLR1	-0,081	-0,165	-0,061	2,265	2,042	1,591
KMO	-0,338	-0,010	-0,287	1,979	1,527	1,174
PTGES	0,154	0,116	0,019	1,954	1,192	1,158
CXCL3	-0,136	-0,008	-0,095	-2,446	-0,046	-1,031
RICTOR	-1,204	-0,159	-0,631	-2,035	-0,308	-0,766
EREG	0,049	0,433	-0,041	-1,819	-1,211	-0,983

Table 2. Continued.

Gene Symbol	Log2 FC in Un-stimulated culture			Log2 FC in LPS-stimulated		
	T1-T0	T2-T0	T3-T0	T1-T0	T2-T0	T3-T0
ZC3H12C	0,020	0,344	-0,114	1,948	1,643	0,715
SKIL	0,226	0,010	0,3023	0,869	1,526	0,598
CXCL10	0,016	-0,056	0,109	1,500	1,432	1,340
IL6	0,313	0,259	0,373	1,063	1,228	1,165
OLIG2	-0,384	-0,563	-0,365	-1,087	-1,499	0,054
C7orf68	-0,539	-0,187	-0,154	-1,008	-1,426	-0,203
CHMP4B	0,6431	-0,138	0,267	1,844	1,212	1,720
JUN	0,431	-0,152	0,560	1,154	0,184	1,562
TNFRSF9	0,063	0,452	0,648	0,967	1,144	1,536
HIVEP1	0,170	-0,106	-0,079	1,826	1,184	1,416
HIF1A	0,370	-0,333	-0,160	0,263	1,077	1,395
PPP1R3B	0,584	0,183	-0,198	-1,145	-0,330	-1,121
MARCH9	-0,309	0,254	-0,039	-0,614	-0,808	-1,118
AXIN2	-0,449	-0,155	-0,886	-0,555	-0,551	-1,094

Table 3. The KEGG pathways significantly over-represented between male and female athletes in LPS-stimulated cultures following exhaustive exercise.

KEGG pathways		**Count**	**Pvalue**
30min post Ex.	Toll-like receptor signaling pathway	7	0.000
	Cytosolic DNA-sensing pathway	4	0.004
3h post Ex.	Cytosolic DNA-sensing pathway	4	0.000
	Toll-like receptor signaling pathway	5	0.001
	RIG-I-like receptor signaling pathway	4	0.001
	Amoebiasis	4	0.004
24h post Ex.	Epithelial cell signaling in Helicobacter pylori infection	3	0.010