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INVESTIGATION OF THE OPPOSING EFFECTS OF ENVIRONMENTAL FACTORS ON THE PREDISPOSITION TO ARTERIAL THROMBOSIS IN A MOUSE MODEL CARRYING THE HUMAN BDFNVal66Met POLYMORPHISM

BIO/14

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A mio nonno Costi

A mia nonna Lina

per avermi fatto dono del loro amore.

"Cuando veas una estrella fugaz

Guárdala en tu corazón

Es el alma de alguien que consiguió

Dar a los suyos su amor"

Mägo de Oz - La Danza del Fuego

Abstract

Background: Despite the growing knowledge and the advances in the prevention and treatment of cardiovascular disease (CVD), this pathology is still the leading cause of morbidity and mortality worldwide. Of note, environmental factors such as stress, depression, and anxiety, were recently included in the category of risk factors alongside the canonical ones since their ability to modulate the onset and progression of CVD and to influence the response to therapies. In this context, the new field of behavioral cardiology aims to reach a deeper understanding of the pathophysiology of behavior-related CVDs and the development of effective interventions both to modify high-risk lifestyles and behaviors and to reduce psychosocial risk factors for patients. Among the non-pharmacological treatments, growing amount of literature shows that physical exercise (PE) takes hold as a clinical management strategy for its positive effect on both psychological pathologies and CVD. Interestingly, the family of proteins named neurotrophins was found to be involved in the patho-physiology of both cardiovascular and nervous system. Among them, the human BDNF Val66Met polymorphism is known to be associated to neuro-psychiatric disorders, anxiety and to higher susceptibility to stress, and recently to the individual predisposition to arterial thrombosis related to acute myocardial infarction (AMI) and to eating disorders and obesity.

Aim of the study: The aim of this study was to highlight the impact of the interplay between BDNF Met allele and positive (physical exercise) and negative (chronic stress) environmental factors on the risk of arterial thrombosis.

Results: Taking advantage of a knock-in mouse carrying the human BDNF Val66Met polymorphism that represents a good model of the pathologies observed in human, we showed that spontaneous physical exercise is able to induce positive morphological changes and reduce the inflammatory profile of the adipose tissue in homozygous BDNF Met/Met mice. These beneficial effects might be at the bases of the observed reduction in the pro-thrombotic phenotype detected in this animal model. In addition, our *in vitro* data well support the role of Pro-BDNFMet in modulating adipogenesis in line with what observed in the epididymal white adipose tissue of BDNF Met/Met mice.

In addition, sub-chronic stress is sufficient to unveil the pro-thrombotic phenotype in heterozygous BDNF Val/Met mice affecting the number and functionality of blood circulating cells, and the expression of key thrombotic molecules in arterial tissue.

Conclusions: This study supports the important interaction between both positive and negative environmental factors and Met allele of the BDNF gene in relation to the modulation of arterial thrombosis. Human studies will be crucial to confirm this possible gene-environment interaction and to assess the necessity of taking this interaction into account to deploy better strategies of clinical management of the arterial thrombosis risk in patients carrying this polymorphism.

Riassunto

Background: Nonostante il progresso nella comprensione dei meccanismi cellulari e molecolari e gli avanzamenti nel campo della prevenzione e dei trattamenti delle malattie cardiovascolari, queste rimangono ancora la principale causa di mortalità e morbidità a livello mondiale. È interessante notare come fattori di rischio ambientale, quali stress, depressione e ansia, siano stati recentemente inclusi accanto ai classici fattori di rischio tradizionali data la loro capacità di modulare l'insorgere e la progressione delle malattie cardiovascolari ed influenzare la risposta alle terapie. In questo contesto si inserisce il nuovo settore della cardiologia comportamentale il cui obiettivo è quello di arrivare a comprendere e approfondire la conoscenza della patofisiologia alla base delle malattie cardiovascolari legate alla sfera comportamentale nonché sviluppare strategie di intervento efficaci al fine di modificare gli stili di vita e i comportamenti ad alto rischio, riducendone così l'impatto sui pazienti. La letteratura degli ultimi anni ha dimostrato come l'esercizio fisico risulti una valida strategia di trattamento a livello clinico risultando efficace sia per il trattamento delle patologie psicosociali che di quelle cardiovascolari. In particolare, è stato osservato come la famiglia di proteine dette neurotrofine sia coinvolta nei processi patofisiologici a carico sia del sistema nervoso che di quello cardiocircolatorio. Tra le neurotrofine, il brain-derived neurotrophic factor (BDNF), ed in particolare il polimorfismo a singolo nucleotide denominato BDNFVal66Met, si sa essere associato a malattie neuropsichiatriche, ansia, maggiore suscettibilità allo stress e recentemente ad una maggior predisposizione alla trombosi arteriosa associata a infarto acuto del miocardio, nonché con disturbi del comportamento alimentare e obesità.

Obiettivo dello studio: L'obiettivo del presente studio è stato quello di mettere in evidenza l'impatto che l'interazione tra la presenza dell'allele Met e fattori ambientali positivi, come l'esercizio fisico, o negativi, come lo stress cronico, può avere rispetto al rischio di sviluppare trombosi arteriosa.

Risultati: Il modello murino knock-in per il polimorfismo umano BDNFVal66Met rappresenta un buon modello per lo studio delle patologie che questa mutazione genera nell'uomo.

Topi omozigoti per l'allele Met ($BDNF^{Met/Met}$) sono stati da noi utilizzato per mostrare come l'esercizio fisico spontaneo sia in grado di indurre cambiamenti positivi nella

morfologia del tessuto adiposo e ridurre l'infiammazione locale. Questi effetti positivi potrebbero essere alla base della riduzione del fenotipo pro-trombotico osservato in questo modello murino. Inoltre, dati *in vitro* sostengono il ruolo del Pro-BDNFMet nella capacità di modulare l'adipogenesi in linea con quanto osservato nel tessuto adiposo epididimale dei topi $BDNF^{Met/Met}$.

In topi eterozigoti per l'allele Met ($BDNF^{Val/Met}$), lo stress sub-cronico è risultato sufficiente per smascherare il fenotipo pro-trombotico portando all'innalzamento del numero e della funzionalità delle cellule del sangue e dell'espressione di fattori chiave per il processo trombotico a livello arterioso.

Conclusioni: Questo studio dimostra un'importante interazione tra fattori ambientali positivi o negativi e l'allele Met del BDNF in relazione alla modulazione della trombosi arteriosa. Studi sull'uomo saranno necessari al fine di confermare questa interazione gene-ambiente e comprovare la possibilità di prendere in considerazione l'interazione al fine di mettere in atto migliori strategie di trattamento clinico del rischio trombotico in pazienti recanti il polimorfismo in oggetto.

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1. INTRODUCTION

1 Risk factors in cardiovascular diseases and thrombosis

Cardiovascular diseases (CVDs) are the general and broad term by which a heterogeneous group of disorders involving the heart and the blood vessels is identified. This group of pathologies comprehends congenital and acquired cardiomyopathies, arrhythmias, myocardial infarction, coronaropathy, anginas, hypertension, peripheral arterial and vascular diseases, stroke and also chronic kidney disease and tumors.

Despite the introduction of new regimens of pharmacological interventions and the continuous progression in the development of implant surgery, surgery techniques and advanced technological devices for diagnosis, CVDs still represent the first cause of morbidity and mortality worldwide. In 2016 an estimated 17.9 million people died from CVDs, accounting for 31% of all global deathsa and over three-quarters of these took place in low- and middle-income countries (WHO 2017, Organization 2019). The Framingham Heart Study represented a milestone in the identification of independent major risk factors for CVDs (Mahmood et al. 2014). After this, a growing amount of literature was able to recognize over 300 risk factors that were afterward categorized into two main groups:

-modifiable factors: like high blood glucose and diabetes mellitus, obesity, high blood cholesterol, high blood pressure. In this category are also found the so-called behavioural risk factors such as smoking habit and alcohol consumption.

-non-modifiable factors: like family history, genetic factors, ethnic group, age, and sex.

It was believed that acting directly or indirectly on modifiable risk factors could represent a winning strategy to reduce the impact of CVDs on population but results were far from what estimated. The following studies tried to comprehend this controversial founding that the risk factors identified so far were not sufficient to reach an adequate accuracy for the comprehension of the causes, and subsequently for the treatment, of CVDs leading to hypothesize that something was still missing (Brotman et al. 2005). It was reported that conventional risk factors for CVDs are predictors of the majority, but not the totality, of morbidity and mortality, raising the awareness on the need to identify new dynamics involved in the onset and progression of these pathologies (Stamler, Wentworth, and Neaton 1986). Starting from this, researchers were able to identify new, non-conventional risk factors that were named “emerging”. Among them, gain or loss of function related

to mutations and single nucleotide polymorphisms (SNPs) of genes known to be involved in cholesterol and fatty acid metabolism such as CEPT (Ference et al. 2017), LDL and HDL (Mega et al. 2015) and PCSK9 (El Khoury et al. 2017) were identified. These studies were subsequently confirmed by Genome-wide association studies (GWAS) and opened the way to new therapeutically approaches (Kessler, Vilne, and Schunkert 2016). At the same time, the measurement of carotid intima-media thickness and blood concentrations of C-reactive protein, myeloperoxidase and F2-isoprostane, vitamin D and apolipoprotein B and lipoprotein(a) were correlated to the presence and the evolution through time of CVDs (Gupta et al. 2013). Interestingly, some researchers suggested that social, economic and cultural changes occurring in the last two decades might account for the major incidence of CVDs (Havranek et al. 2015). In particular, it was shown that, along with the increase in CVDs, there was also a growing trend in the diagnosis of psychological and psychosocial disorders (Cohen and Janicki-Deverts 2012, Brody 2018). Starting from this evidence, different research groups focused their attention on the possible relationship between stress or depression and the development of CVDs, showing. a causal relationship between these two groups of pathologies (Strike and Steptoe 2004, Steptoe and Kivimäki 2012, Jiang, Krishnan, and O'Connor 2002, Cohen, Edmondson, and Kronish 2015). Interestingly, both stress and depression contribute directly to CVD disease via different mechanisms inducing endothelial dysfunction, myocardial ischemia, plaque rupture, arrhythmias and thrombosis (Bairey Merz et al. 2002, Huffman et al. 2013). Moreover, chronic stress is reported to have a direct causative role in the onset of major depression (Yang et al. 2015) and CVDs (Joynt, Whellan, and O'Connor 2003).

1.1 Key regulators of thrombosis

Haemostasis and thrombosis are two opposite physiological processes cooperating in a delicate equilibrium to:

-keep the blood in a fluid state

-prevent abnormal and uncontrolled haemorrhage

-maintain vascular integrity through the correct timing of formation and lysis of blood clots.

Different cells participate in the balance of these processes such as platelets, red and white blood cells, and endothelial cells, interacting with different proteins that

behave as thrombotic and anti-thrombotic factors. However, in pathological conditions, this delicate balance is disrupted leading to opposite conditions characterized by excessive bleeding (as happens in hemophilia, anemia, liver cirrhosis, leukemia, and vitamin K deficiency) or formation of big and stable thrombi that lead to vessel occlusion. In this case, thrombus formation is the triggering event of several vascular diseases such as myocardial infarction (MI), cerebrovascular thrombosis (often referred to as stroke or cerebral ictus) and venous thromboembolism (VTE). Even if both characterized by the formation of a blood clot, venous and arterial thrombosis are two highly distinct pathological conditions.

Venous thromboembolism (VTE) is the general term to indicate in a single word both deep-vein thrombosis (DVT) and pulmonary embolism (PE). This condition is prevalent in large and deep veins of the legs. The clots forming in this condition are referred to as red thrombi since they are primarily composed of red blood cells and fibrin. In particular, they form due to defects of the proteins involved in the coagulation and fibrinolysis cascade or secondary hypercoagulable states, involving abnormalities of blood vessels and blood flow as happens in cancer, obesity or after major surgery (Cushman 2007, Urbach et al. 2003).

Arterial thrombosis is characterized by the formation of clots called in this case white thrombi. Arterial thrombi are platelet-rich and their formation reflects vessel wall defects and its consequence (Freedman 2005). For example, the primary cause of arterial thrombosis is either instability or rupture of an atherosclerotic plaque resulting in localized clot formation and blockage of blood flow with subsequent MI or stroke. Hypertension, hyperlipidemia, smoke habit and diabetes mellitus are well-known and characterized risk factors for arterial thrombosis (Owens and Mackman 2010). During the onset and progression of these pathological conditions, a series of interacting processes involving vascular wall, platelets, leukocytes, and coagulation proteins take place. Haemostasis is driven primarily by the coagulation system with the expression of tissue factor and besides that by platelet activation, contributing to the haemostatic plug formation and the reinforcement of the coagulation system (Versteeg et al. 2013).

1.1.1 Role of platelets and vessel wall

Platelets (PLTs) are the smallest cells in the human body, with a diameter of 2-4µm that are produced daily by fragmentation of their progenitors, the megakaryocytes. For their origin, platelets lack the nucleus and genomic DNA (Italiano and

Shivdasani 2003) but still have mRNA and all the transcription machinery necessary for the translation process to synthesize proteins (Nieswandt, Varga-Szabo, and Elvers 2009). Since their first observation, it was understood that PLTs have an essential role in the thrombotic process and nowadays their importance is well established also in inflammation, immunity, atherosclerosis (Nording, Seizer, and Langer 2015) and mental-psychiatric disorders (Ehrlich and Humpel 2012).

Platelets contribute to the thrombotic process through their ability to adhere to the damaged vessel wall thus becoming in this way activated and able to secrete several components and to aggregate. Primary hemostasis starts with discoid PLTS adhering to the sub-endothelial matrix via the interaction between specific adhesive glycoproteins (GPs) on their surface and extracellular matrix (ECM) proteins.

In particular, the most important interaction between platelets and ECM proteins is mediated by the multimeric plasma protein von Willebrand factor (vWF). vWF associated with both the major matrix protein collagen, the substrate of platelet adhesion predominantly under high shear, and with the GP Ib/IX/V complex expressed on platelets. Among the collagen types expressed in the ECM, vWF has a major affinity for type I and III. Circulating vWF has a structure that prevents its interaction with GP complex located on platelets surface; however, after collagen binding, it undergoes a shape change exposing different sites that are recognized by the GP Ib/IX/V complex, thus promoting platelets adhesion to ECM.

GPIba is the first one interacting with vWF and it is responsible for platelets immobilization to the lesion site; after this, other GPs take part in the activation and stabilization processes (Savage, Almus-Jacobs, and Ruggeri 1998). It was showed that GP Ib/IX/V complex, after mediating the interaction with vWF, also binds to other adhesive proteins such as collagen, thrombospondin-1, α-thrombin, and coagulation factors (Rivera et al. 2009). Despite the fundamental role of collagen-vWF-GPIb/IX/V in the platelet adhesion process, many other interactions play a crucial role in platelet immobilization and activation in the lesion site. Also, integrin α2β1 (GPIa/IIa) is able to bind collagen and this process represents one of the first steps of PLTS adhesion, even if it is not understood if GPIa/IIa alone is able to drive this process (Munnix et al. 2008). Other GPs such as αvβ3, integrin α5β1, and integrin α6β1, even if not highly expressed on platelets surface, contribute to these processes, binding other adhesion proteins including fibronectin, vitronectin, laminin and Thrombospondin-1 (Shattil and Newman 2004). Although the great

amount of fibronectin is detected in plasma, megakaryocytes, and α-granules of platelets (Maurer, Tomasini-Johansson, and Mosher 2010), its role in thrombus formation and stability is limited (Maurer, Tomasini-Johansson, and Mosher 2010, Ni et al. 2003). Similarly, it is still unclear the role of plasma and endothelial extracellular matrix (ECM) vitronectin during platelet activation (Preissner and Reuning 2011). However, it is well known that vitronectin is indirectly involved in the coagulation stabilizing plasminogen activator inhibitor-1 (PAI-1) and then reducing the clot lysis (Zhou et al. 2003). Laminin, synthesized by endothelial cells, is present in the ECM and in the basement membrane, and it is exposed after mild vascular injury (Hamill et al. 2009), supporting PLTs adhesion to the subendothelium. Finally, Thrombospondin-1 is released from α-granules of platelets and binds to CD36, sustaining platelet activation (Jurk et al. 2003).

These protein-protein interactions induce activation of PLTs promoting morphological changes characterized by shape change with the protrusion of plasma membrane pseudopodia originating from changes in the cytoskeleton (Fox 1993). During these events, the granules present in the PLTs fuse with the membrane releasing their content by exocytosis. Among the released factors adenosine diphosphate (ADP) and serotonin, named secondary agonists, have the ability to potentiate the stimulation of other platelets, which are attracted to the lesion site. ADP is an amplifier of platelet activation and two of its receptors can be found on their surface (Gachet 2001). The P2Y1-receptor is involved in Ca^{2+} mobilization in the platelet favouring shape change and the transient aggregation (Fabre et al. 1999) while the P2Y12-receptor potentiates platelet secretion activity and it is involved in sustained irreversible aggregation (Dorsam and Kunapuli 2004). Serotonin acts as an amplifier of platelet response acting as a mediator for the retention of procoagulant proteins like fibrinogen and thrombospondin on the platelet surface (Dale et al. 2002). An important role is also played by thromboxane A2 (TXA_2), which is an agonist released by activated platelets supporting both autocrine (self) and paracrine (adjacent) platelet activation, and recruiting the surrounding platelets to the site of the growing thrombus (Riccioni et al. 2007, Roberts, Vaziri, and Barnard 2002). TXA_2 in humans stimulates two subtypes of G-proteins couples named TPα and TPβ, even if the effects in platelets are mediated predominantly through the α isoform (Murugappan, Shankar, and Kunapuli 2004) by the activation of adenyl cyclase activity (Davì et al. 1997).

Activated platelets start their bridging thanks to the interaction between their surface receptor GPIIbIIIa and its ligand fibrinogen starting in this way the aggregation process. This interaction determines conformational changes in GPIIb/IIIa facilitating the stabilization of platelet-platelet aggregates and giving GPIIbIIA the ability to bind other proteins such as vWF, fibronectin, vitronectin, and CD40L thus maintaining clot firmness. At this point, platelet plug undergoes the formation of insoluble cross-links mediated by the conversion of fibrinogen to fibrin by the activated coagulation factor thrombin. This process is called secondary hemostasis and during this, platelet amplification of the stimulation signal leads to their ability to bind clotting factors (procoagulant activity) by the interaction with specific high-affinity binding sites and the formation of a stable platelet-fibrin plague with subsequent clot retraction (Jurk and Kehrel 2005).

1.1.2 The coagulation cascade

The model of coagulation cascade was developed in the 60s when it was understood that each clotting factor consists of a pro-enzyme that is converted to its active enzymatic form by another upstream-activated factor (DAVIE and RATNOFF 1964, MACFARLANE 1964). It is now well understood that there are two different cascades named intrinsic pathway, called in his way since all the interacting molecules are present in the blood, and extrinsic pathway, in which tissue factor (TF) necessary to start the process is coming from extravascular tissue. However, both pathways converge on the activation of FX. Following vascular damage, sub-endothelial and smooth muscle cells are exposed to the bloodstream thus showing TF on their surface. TF interacts with factor VII and upon activation TF/FVIIa complex is able to convert FIX and FX into FIXa and FXa, respectively. This allows FXa to associate with cofactor FVa to form a prothrombinase complex on TF-expressing cells. The amplification of the coagulation cascade promotes the conversion of prothrombin (FII) into thrombin (Monroe, et al., 2006). The slowly accumulating amounts of thrombin will further activate platelets that adhered to the site of injury. In parallel, thrombin converts platelet-derived FV into FVa, thus amplifying prothrombinase activity, and converts FVIII into FVIIIa, which acts as a cofactor to FIXa on the surface of activated platelets to support FXa generation. In addition, thrombin converts FXI into FXIa. Activated FXI converts FIX into FIXa, which then associates with thrombin-cleaved FVIII. On phosphatidylserine-exposing cell membranes, the tenase complex of FIXa/FVIIIa catalyzes the conversion of FX

to FXa, after which the FXa/FVa complex produces sufficient amounts of thrombin to massively form fibrin fibers. As a final step, the thrombin-activated plasma transglutaminase FXIIIa catalyses the formation of a covalent crosslink between adjacent fibrin chains to yield an elastic, polymerized fibrin clot.

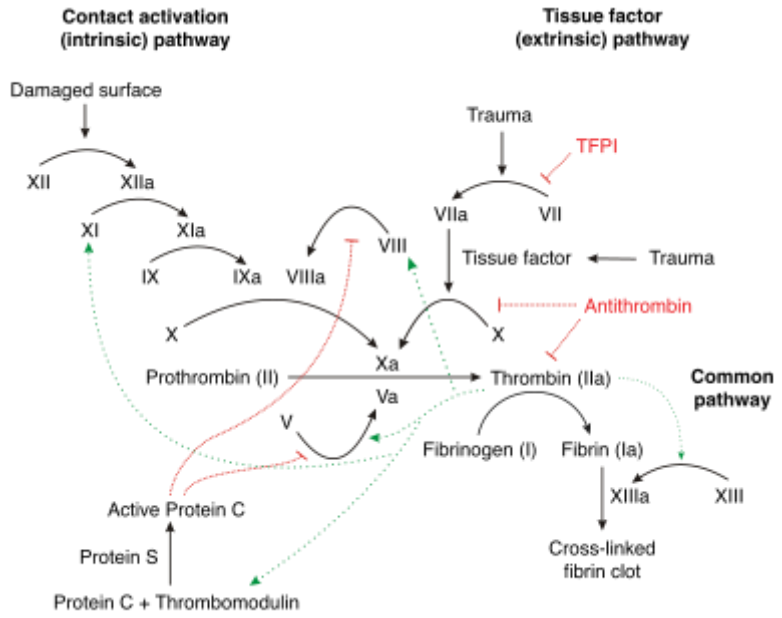


Figure 1. Schematic representation of the model of the coagulation cascade.

Concomitantly with the coagulation cascade, a fundamental role is played by the negative control of coagulation by which the system controls the spreading of the clot formation (Broze et al. 1988). Different proteins are involved in this system. Antithrombin acts inhibiting FIXa, FXa, and thrombin, heparin cofactor II inhibits FIIa, and TFPI reversibly inhibits coagulation by direct binding to free FXa and by interaction with the transient TF/FVIIa/FXa complex (Girard et al. 1989). The enzyme-based protein C/protein S pathway acts inactivating FVIIIa and FVa by protolithic cleavage, thus suppressing tenase complex and prothrombinase actions providing another anticoagulant modality.

In particular, Tissue factor (TF), also named CD142 or thromboplastin, is a transmembrane glycoprotein of about 47kDa and in its mature form, it is constituted by 219 amino acids in the extracellular region, a transmembrane region and a short tail (21 amino acids) in the intracellular side. The outside domain is formed by two fibronectin type III domains, each with an extracellular disulphide bond (Versteeg et al. 2013).

TF is expressed on the surface of fibroblasts and smooth muscle cells and its localization is fundamental since it acts as a haemostatic barrier upon vessel injury. TF is also expressed by circulating monocytes and tumour cells that may initiate pathological conditions such as disseminated intravascular coagulation (Rickles, Patierno, and Fernandez 2003). TF may be found also in microparticles released by the cells (Giesen et al. 1999), representing the so-called blood-born TF. Nowadays the opinion is that TF expressed by cells in the vessel wall is about 1000-fold the one circulating in the blood (Butenas et al. 2005) thus making its role in thrombosis a subject of debate.

It was demonstrated that platelets contain TF pre-mRNA that is converted into mature mRNA and might in this way generate a small amount of protein upon platelets activation (Schwertz et al. 2006, Panes et al. 2007). In addition, it was observed that Meg-01 megakaryoblastic cell line express TF and that it is transferred to a subset of shed platelet-like particles (Brambilla et al. 2015). Moreover, TF was found to be expressed after platelet activation (Camera et al. 2003, Brambilla et al. 2008) and in particular localises on the biggest activated ones (Camera et al. 2012). In line with these data, also platelets can be a source of active “blood-borne” TF, which can sustain activation of the blood coagulation on the edge of a growing thrombus. However, some authors showed that activated PLTS, after P-selectin exposure, are able to bind TF-bearing microvesicles deriving from monocytes thus leading to the formation of highly thrombogenic platelet-microvesicles hybrid. (Del Conde et al. 2005, Falati et al. 2003, Bouchard, Mann, and Butenas 2010, Østerud 2003, Owens and Mackman 2011).

Alongside its action in thrombosis, TF was found to participate in the migratory and proliferating ability of smooth muscle cells (Pyo et al. 2004) and to be involved in cancer-related vascularization and metastasis (Steffel, Lüscher, and Tanner 2006). However, it was also demonstrated that TF is expressed as a response to inflammatory stimuli as chemokines or cytokines (Erlich et al. 1999).

Among the inflammatory molecules responsible for TF induction there are tumor necrosis factor-α (TNF-α), interleukin-1β or CD40 ligand (Bavendiek et al. 2002) but also biogenic amines such as serotonin (Kawano et al. 2001) or histamine (Steffel et al. 2005), and mediators such as thrombin, oxidized LDL or vascular endothelial growth factor (Camera et al. 1999).

1.1.3. Leukocytes

The presence of the leukocyte population participating in the thrombotic process was observed at the end of the XIX century but their role is still under debate. In particular, it seems that aside from their canonical inflammatory role, the prevalent role of leukocytes in thrombus formation is to assign to the interaction with PLTs thus forming mixed aggregates (Cerletti et al. 2012).

Leukocytes are able to bind either with activated circulating PLTs or with the one's adherent to the site of vessel wall injury (Gawaz, Langer, and May 2005, Weber and Springer 1997). Leukocytes initially adhere to PLTs via PSGL-1-P-selectin interactions (Evangelista et al. 1999, Yang, Furie, and Furie 1999) and this adhesion is subsequently stabilized by the binding between Mac-1 and GPIbα (Simon et al. 2000). These interactions with the receptors PSGL-1 and Mac-1 on the monocyte surface are able to induce the inflammatory cascade enhancing the secretion of effector molecules such as CCL5 (Weyrich et al. 1996, Neumann et al. 1997). Lymphocytes are able to interact with PLTs based on the binding between P-selectin-, CD154- and αIIbβ3 (Hu et al. 2010). Different studies suggested that platelets-leukocytes complexes are more prone to interact with endothelial cells thus enhancing in-site inflammation that can cause an accelerated progression of atherosclerosis (Huo et al. 2003, Lievens et al. 2010). In addition, high levels of neutrophil and platelet-monocyte complexes were correlated with acute myocardial infarction (Furman et al. 2001). Besides their role in forming complexes with PLTs, leukocyte can have a direct role in the thrombotic process through the release of tissue factor (TF), the initiator of blood coagulation. In particular, following stimulation with lipopolysaccharides (LPS) and C-reactive protein, TF mRNA is highly translated to the mature protein and exposed to monocyte membrane (Sovershaev et al. 2007). TF could stimulate differentiation of monocyte to macrophages thus enabling their migration through the arterial wall and promoting their accumulation in atherosclerotic plaques, as shown in human and mouse tissues (Lambert, Sachais, and Kowalska 2007, Muhlfelder et al. 1999).

Monocytes/macrophages, synthetizing interstitial collagenase (MMP-1) and stromelysin (MMP-3) through CD40 stimulation, participate in plaque destabilization and rupture key processes the atherothrombotic events (Mach et al. 1997).

Finally, monocytes not only take an active part in the coagulation, but they also support it reducing the fibrinolysis. Indeed, resting naïve monocytes produce

thrombomodulin (Satta, Freyssinet, and Toti 1997), an essential cofactor of thrombin in triggering the natural anticoagulant protein C pathway.

1.1.3 Fibrinolytic system and control of thrombosis

The fibrinolytic system represents, along with primary and secondary haemostasis, the crossroad between haemostasis and thrombosis since its balanced regulation is fundamental on one side to avoid haemorrhages and on the other one to prevent thrombus excessive growth. The process is mediated by two plasminogen activators (PAs), the tissue-type PA (tPA) and the urokinase-type (uPA) acting through a cellular u-PA receptor (u-PAR). These activators convert plasminogen to plasmin that degrading fibrin and activating matrix metalloproteinases (MMPs) promote clot lysis and extracellular matrix degradation (Collen and Lijnen 1991, Birkedal-Hansen 1995). These processes are counteracted by the inhibitors of PAs (PAI-1 and PAI-2) that prevent plasminogen to plasmin conversion and by α2-antiplasmin that inhibits directly plasmin. The fibrinolytic system could be impaired by an alternated t-PA release from the vessel wall or by increased rates of the neutralization system. Regarding this latter case, it was observed that PAI-1 plasma concentration is enhanced in different pathological condition including venous thromboembolism, obesity, sepsis, and CAD. On the other hand, increased levels of tPA or deficiency of α2-antiplasmin or PAI-1 are associated with a bleeding tendency (Collen 1999).

2 Psychological stress and CVDs

The term stress was coined by Hans Selye in 1936 to describe “the non-specific response of the body to any demand for change” (Selye 1936). This definition resulted from the observation that different acute ambient stimuli (named stressors) were all able to induce three main pathological conditions: hyperaemia and enlargement of the adrenals, atrophy of the thymus and lymph nodes as well as hemorrhagic gastric erosions/ulcers (stress triad). Further studies in the last eighty years showed that in addition, if these stressors were protracted, they could induce chronic alterations leading to heart attacks, stroke, kidney disease, and rheumatoid arthritis (Dimsdale 2008, Bruce, Griffith, and Thorpe 2015, Walker et al. 1999).

However, it must be underlined that stress is a necessary response that the body puts in place to overcome a new or difficult situation when a higher efficiency and productivity is needed. For this reason, heart rate and respiratory acts accelerate, blood pressure increases, sweating intensifies to maintain under control body temperature and appetite is reduced but energy metabolism becomes more efficient.

When transient, acute stress, these alterations are easily handled by the body that immediately after the cessation of the stressor(s) returns quickly to homeostasis. It is the one resulting from specific events or situations that are perceived by the body as completely new or dangerous (Bryant 2018).

However, when the stressful events are close to one another or when the stressors become chronic (chronic stress), then the organism is not able to compensate for the situation and to return the systems in the homeostatic situation.

A population study showed that, under a chronic stressful condition, patients that already had a myocardial infarction showed a 2.5x incidence of a second event than not stressed ones. The same study analysed data regarding 3335 people affected by coronaropathy, identifying a correlation with stress with a 4.4x risk increase (Bosworth et al. 2000). A longitudinal study performed on the analysis of data regarding 7066 women and men from the Copenhagen City Heart Study showed a causal relationship between stress and cardiovascular diseases mediated through unfavourable changes in health behaviour and cardiac risk profile (Rod et al. 2009).

Regarding the association between stress and CVDs, it was demonstrated that there are at least three macro-areas in which we can classify it: the impact of work stress, marital and domestic stress and social support.

Regarding work stress, it is estimated that up to 40% of all workers are stressed and in a third of these, the condition is chronic (Strike and Steptoe 2004, Peter and Siegrist 2000). An analysis performed among 13 different studies, showed that in 10 of them an association between work stress and CVDs could be found, while in the other three not significant score was reached (Kuper, Marmot, and Hemingway 2002). Work stress is showed to increase the incidence of coronary artery disease (CAD) and, in patients already hospitalized for CAD, it increases the risk of new events (Aboa-Eboulé et al. 2007).

Marital and domestic stress is showed to worsen the prognosis in women with progress CVDs (Strike and Steptoe 2004) and taking care of the sick husband independently from other canonical risk factors such as smoke, age, hypertension and diabetes (Lee et al. 2003). A Swedish study found that, after adjusting for classic risk factors, a cohort of women having both work and marital-domestic stress enhances the risk of CVDs up to 5 times versus the control cohort (Orth-Gomér et al. 2000). Domestic stress has a major impact on people with a low socio-economic status in a relationship with the level of instruction and work position (Rose and Marmot 1981). In support of these results, in countries with low social support, there is an increase in behavioral risk factors such as smoking, unbalanced diet and low physical activity (Rosengren, Orth-Gomér, and Wilhelmsen 1998), but also stress levels are higher and associated with enhanced risk of CVDs (Strike and Steptoe 2004).

All these evidences from literature lead to consider the role of lifestyle behaviour on CVDs and to coin the term “behavioural cardiology” as “*an emerging field of clinical practice based on the recognition that adverse lifestyle behaviours, emotional factors, and chronic life stress can all promote atherosclerosis and adverse cardiac events*” (Rozanski et al. 2005). In particular, the role of the new discipline would be a deeper understanding of the pathophysiology of behaviour-related CVDs and the development of effective therapeutic interventions both for modifying high-risk lifestyles and behaviours and for reducing psychosocial risk factors for patients. Unfortunately, until now there have been few large scale trials on the effect of both classical, as for example antidepressant drug use, and alternative, as physical exercise training, treatments among patients with distress and CVDs. The main problem found was the lack of patient's adherence to behavioural interventions and the difficulty to find standardized parameters to define distress in people already

suffering from CVDs (Rozanski, Blumenthal, and Kaplan 1999). In addition, the field of behavioural cardiology requires new figures of physicians that could incorporate both cardiology and psychological expertise in clinical practice.

2.2. Biological effects of stress

As above-mentioned, stress determines the activation of the HPA-axis and the release of glucocorticoids (GCs) and catecholamines thus exercising its influence on different body districts. Of note, hyper-activation of the HPA-axis and the subsequent pathological alterations were found in about 70% of depressed patients (Holsboer 2000) suggesting a possible correlation between stress and major depression (Yang et al. 2015). For example, it was demonstrated that a constant stimulation by cortisol not only can lead to the impairment of the molecular pathways above mentioned but can also have a direct role in neuron excitation. Under normal conditions, GCs contribute to the termination of the stressful reaction via feedback loops that involve the participation of the hippocampus and paraventricular nucleus (Berton and Nestler 2006, Pariante and Lightman 2008). However, constant cortisol and GCs stimulation is able to induce corticosteroid-resistance and decreased function of the glucocorticoid receptor (GR) (Pariante and Lightman 2008). High cortisol level was found able to induce excitotoxicity to pyramidal neurons in the hippocampus leading to spine loss and atrophy of dendrites (Berton and Nestler 2006) and reduced hippocampal volume (Manji, Drevets, and Charney 2001), conditions that are common also in patients affected by major depressive disorder (Yang et al. 2015). Furthermore, it was demonstrated that HPA-axis hyperactivation is directly related to an alteration in the levels of vasopressin (Hodgson et al. 2014), brain-derived neurotrophic factor (Duman and Monteggia 2006), glial cell-derived neurotrophic factor (Uchida et al. 2011), and neurotransmitter system such as glutamate and its receptors (Hashimoto 2011), NMDA, AMPA and metabotropic glutamate receptors (Berton and Nestler 2006), gamma-aminobutyric acid (Möhler 2012) and serotonin (Berton and Nestler 2006). Of note, all these alterations are considered molecular signatures related to the development and progression of major depressive disorder. A recent meta-analysis (Park et al. 2019), showed an association between psychosocial stress, major depressive disorder, and epigenetic changes. Interestingly the alterations that were found are relative to the glucocorticoid signaling (NR3C1 and FKBP5), the serotonergic signaling (SLC6A4),

and the neurotrophin system (BDNF) clearly showing the tight relationship between the alterations induced by stress and major depression. In addition, it was observed that likely in stressed people, also depressed patients present increased levels of pro-inflammatory cytokines that are known to be involved in the reduction of monoamine levels and so in the modulation of cognition, sleep and reward processes (Karrenbauer et al. 2011, Wong and Licinio 2001). Elevated levels of IL-1β are found in depressed patients and they are able to activate the HPA-axis in the same way as stress does, suppressing hippocampal long-term potentiation and down-regulating BDNF expression (Koo and Duman 2009). Mice lacking IL-6 are protected from depressive symptoms induced by stress (Chourbaji et al. 2006) and have not an impairment in neuron proliferation (Koo and Duman 2009). Also, tumor necrosis factor-α (TNF-α) is able to activate HPA axis (Kaster et al. 2012), as well as interleukin-1β (IL-1β) that is able to promote the serotonin uptake suggesting that its blockade could be helpful in depression treatment (Zhu, Blakely, and Hewlett 2006). As a proof of concept deletion of either TNF-α receptor 1 (TNFR1) or TNFR2 generates animal models resistant to induced depression (Simen et al. 2006) while TNF-α injection induced depression-like phenotype which could be prevented by antidepressant drugs (Kaster et al. 2012). However, it is well known that psychosocial and psychiatric pathologies are strictly related to the environment and to the psychological history of each individual and so these parameters, even in presence of common cellular and molecular features between stress and depression, must be taken into account before demonstrate the unidirectional association between these two pathologies. Different studies are available regarding the dynamic relationships between stress and depression over time, including for example the effects of childhood and lifetime stress exposure on later reactivity to stress and development of major depressive disorder. Unfortunately, the lacking of common evaluation parameters often biases the results that were obtained leaving uncertainty on the argument (Hammen 2005).

2.1 Biological mechanisms by which psychological stress could predispose to CVDs

Starting from the association observed between psychosocial stress and CVDs observed in clinical studies, basic research tried to understand the molecular mechanism at the crossroad between these pathologies (Strike and Steptoe 2004,

Dhar and Barton 2016). As already said, a stressful event determines an altered response in different organs and systems of the organism thus influencing different biological pathways and leading to pathological alterations (Rosengren et al. 2004, Huffman et al. 2013). Different systemic alterations have been investigated until now, showing a clear contribution of:

-hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis
-hypertension
-immunity response
-endothelial dysfunction
-platelet activation
-coagulation factors
-neurotrophin system

2.1.1 The hypothalamic pituitary adrenal (HPA) axis dysfunction

One of the first observations done by Hans Selye regarding the effect of chronic stress on living beings was the enhanced dimension of the adrenal glands (Selye 1936). After decades of investigations, it is now clear that stress is able to activate the hypothalamic-pituitary-adrenal (HPA) axis with a consequent stimulation of the sympathetic nervous system and increased circulating levels of cortisol and catecholamines (Rozanski, Blumenthal, and Kaplan 1999, Dinan 1994, Banki et al. 1992, Brown, Varghese, and McEwen 2004).

In response to a stressful event, cortical areas of the brain are activated and, through the limbic system, the signals arrive at the hypothalamus. Under neurotransmitter stimulation, cells located in the paraventricular nucleus (PVN) are activated to synthesize and secrete corticotropin-releasing factor (CRF) (Gu, Tang, and Yang 2012). This factor enters the hypothalamic portal venous system and stimulates the corticotrophs located at the anterior pituitary gland to synthesize proopiomelanocortin (POMC) (Pariante and Lightman 2008) that is subsequently cleaved in adrenocorticotropic hormone (ACTH) and alpha-melanocyte-stimulating hormone (α-MSH). CRF from parvocellular neurons also stimulates the release of arginine vasopressin (AVP) from PVN, which together with CRF synergistically stimulates the release of ACTH (Hasan et al. 2012). ACTH stimulates fasciculate and reticularis zones of the adrenal cortex to produce and release glucocorticoids (GCs, cortisol, and corticosterone in human and rodent, respectively) (Hasan et al.

2012), which together with catecholamine released by sympathetic nervous system (SNS) are the main stress hormones (Gu, Tang, and Yang 2012). GCs exert their effects on multiple aspects of the brain function, such as survival of neurons, neurogenesis, hippocampal size and emotional events, and the peripheral functions including metabolism and immunity (Pariante and Lightman 2008). By binding to glucocorticoid receptors (GRs) in the hypothalamus, the pituitary and the medial prefrontal cortex (mPFC), which will result in a decrease in CRF secretion and subsequent reduced release of ACTH from the pituitary, these GCs inhibit activity of HPA axis through negative feedback mechanism to sustain homeostasis (Pariante and Lightman 2008, Gu, Tang, and Yang 2012).

Salivary cortisol is considered the biomarker of the stressful condition in organisms (Hellhammer, Wüst, and Kudielka 2009, Strike and Steptoe 2004).

Cortisol acts as an anti-inflammatory hormone (Yeager, Pioli, and Guyre 2011), however, during acute stress, the high concentrations of cortisol stimulate the hypothalamus, hypophysis (also called pituitary gland) and other non-specific brain regions mediating its effects and thus acting with a negative feedback loop on the HPA axis. High levels of the hormone inhibit the synthesis and release of the corticotropin-releasing hormone (CRH) and of the adrenocorticotropic hormone (ACTH).

During the chronic stress response, cortisol is secreted continuously thus determining a cortisol-resistance and a reduction in the negative feedback loop of the HPA. Cortisol and CRH were found able to induce endothelial dysfunction, thus participating in the onset and progression of plaque formation/rupture and coronary artery thrombosis (Wirtz et al. 2006).

Interestingly, patients affected by the Cushing's syndrome that is characterized by hypercortisolism, frequently show a hypercoagulable and thrombotic condition, thus confirming the role of cortisol over-stimulation in the onset and progression of cardiovascular disease (Erem et al. 2009).

If the activation of HPA response is necessary during acute stress (Wirtz et al. 2006), its hyperactivation (chronic stress response) determine an alteration of the balance between sympathetic and parasympathetic activity with consequent dysregulation of blood pressure, inflammation, platelet activation, plaque rupture, coronary artery spasm and ventricular arrhythmias that could evolve in ventricular fibrillation and cardiac arrest (Rozanski, Blumenthal, and Kaplan 1999). Interestingly, it was found

that the hyperactivation of the HPA axis can influence cerebral functionality causing other hormonal, inflammatory and neurotransmitter alterations that are able on their own to reinforce HPA axis stimulation (Grippo and Johnson 2002).

2.1.2 Psychosocial stress and hypertension

Different studies suggest that stressful events are related to the development of hypertension. Acute stress is able to increase blood pressure by enhancing cardiac output and the heart rate without affecting total peripheral resistance. In addition, the activation of the HPA-axis not only increases the levels of catecholamines and cortisol but has an effect also on vasopressin, endorphins, and aldosterone. All these alterations may in part explain the increase in blood pressure (Zimmerman and Frohlich 1990). It was demonstrated that people experimenting with work stress and low socioeconomic levels have enhanced blood pressure, higher heart rate, and peripheral resistance, especially in the morning (Schnall et al. 1998). Interestingly, two systematic reviews, one of cohort studies and the other on observational studies, clearly showed that acute stress is able only to enhance blood pressure acutely, while several, but not all, the studies included indicate that chronic psychosocial stress is able to induce hypertension (Sparrenberger et al. 2009, Gasperin et al. 2009). However, if the association between chronic psychosocial stress and hypertension is confirmed, no consistent data are available regarding the molecular mechanisms underlying this interaction. It is hypothesized that the involvement of the sympathetic nervous system response and the release of catecholamines leads to increased heart rate, cardiac output, and blood pressure. If sympathetic responses to acute stress are well studied and demonstrated, the process by which this system could sustain blood pressure enhancement leading to hypertension is not well understood. It is supposed that the key could be the prolonged activation of the system and the failure to return to homeostasis following multiple stressful events (Spruill 2010). However further studies are necessary to deepen the understanding of the molecular bases of these processes.

2.1.3 Psychosocial stress and the immune system

It was recently reported that stress can activate the inflammatory response not only in the brain but also in peripheral tissues (Rohleder 2014, Calcia et al. 2016)

As above-mentioned, the activation of the HPA-axis mediates the release of cortisol for which an anti-inflammatory action is known. Of note, upon stimulation adrenal

glands release other hormones that, like cortisol, are part of the class of GCs that are known for their immunosuppressive and anti-inflammatory activity.

The presence of several inflammatory cytokines such as TNF-α and IL-6 stimulates the pituitary-adrenal axis to release GCs that in turn enhance the expression of anti-inflammatory cytokines such as IL-10, TNF-β (Sorrells et al. 2009). However, it was discovered that persistent high levels of GCs increase the expression of the inflammasomes system through the enhanced secretion of IL-1β (Busillo, Azzam, and Cidlowski 2011), CRP, IL-6, TNFα and the transcription factor of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Miller, Maletic, and Raison 2009). In addition, enhanced release of norepinephrine (NE) could regulate the immune and inflammatory system through the secretion of inflammatory factors as transforming growth factor-β (TGF-β) and TNFα as demonstrated in the cell line RAW264.7 (Zhou, Xu, and Jiang 2008). Intriguingly, the presence of a chronic low-grade inflammatory could be a possible mechanism linking chronic stress and CVDs. It was observed that some inflammatory cytokines like CRP, IL-6, TGF- β1 and TNF- α are involved in the onset and progression of thrombotic events and atherosclerosis. In particular, CRP and IL-6 are considered potentially predictive markers of atherosclerosis (Nadrowski et al. 2016). NE, through activation of α adrenoreceptors, could increase the levels of TGF-β1 and TNFα production in macrophage (Huang et al. 2012) and it is showed that these cytokines are related to endothelial dysfunction and renin-angiotensin system (Li et al. 2012). In summary, stress, hyper-activating the HPA-axis induces a constant release of GCs and NE that are known to promote the release of CRP, IL-6, TGF- β1 and TNF- α, inflammatory cytokines well know for their role in the onset and progression of CVDs.

2.1.4 Psychosocial stress and platelets

A large number of modifiable and not-modifiable risk factors such as age, serum cholesterol level, diabetes, catecholamine levels, cigarette smoke, obesity, and alcohol consumption, are well known to be associated with CVDs. A mechanism by which all these risk factors can lead to CVDs is their ability to enhance platelet reactivity. It is well documented that patients with CVDs have hyper-reactive platelets that expose higher levels of P-selectin and GPIIbIIIa thus starting the formation of platelet-leukocyte and platelet-fibrinogen-vWFactor aggregates,

respectively (Merten et al. 2000, Konstantopoulos et al. 1998). In addition, activated platelets release more TXA-A2 and other prostaglandin metabolites (Falk 1985, Fitzgerald et al. 1986) and produce less nitric oxide (Freedman et al. 1998). During acute coronary syndromes, the thrombus formation is driven by the ability of platelets to aggregate with neutrophils and leukocytes that in turn can enhance platelet aggregation (Faraday et al. 2001). Activated platelets produce mediators of inflammation such as platelet-derived growth factor (Selheim et al. 2000), platelet factor 4 (Leavitt 2007) and TGF-β (Grainger et al. 1995).

The release of catecholamines due to the activation of the HPA-axis induced by stress is able to stimulate alpha2-adrenergic receptors (von Känel and Dimsdale 2000) and research data suggest that this is sufficient to enhance platelet activity, reactivity and immune-modulatory capacities (Koudouovoh-Tripp and Sperner-Unterweger 2012). Stressful mental tasks are able to increase acutely the release of PF-4 and TGF-β from platelets (Patterson et al. 1995) and also a significant increase in platelet-leukocyte aggregates formation was observed (Hamer et al. 2006, Steptoe et al. 2003). Three cross-sectional studies reported that stress is able to increase the percentage of platelet-leukocyte aggregates by 15% and the percentage of P-selectin by 5-fold (Aschbacher et al. 2008, Aschbacher et al. 2007, Aschbacher et al. 2009).

2.1.5 Psychosocial stress and coagulation cascade

In the last five decades it was clearly demonstrated that stress is certainly associated with hypercoagulability through the activation of the coagulation system, platelets hyper-reactivity and reduced fibrinolysis (von Känel et al. 2001).

In particular, it was observed that even a single bout of stress is able to enhance the activity of clotting factor VIII, tissue-type plasminogen activator (t-PA) with a concomitant increase in D-dimer, indicating enhanced fibrin turnover. Interestingly, it was found that FVIII:C, as well as fibrinogen and von Willebrand factor (VWF) levels, are increased between 5% and 10% from baseline in healthy subjects in response to acute stress, and that after 20-45 minutes their levels return to pre-stress condition (von Känel 2012, von Känel et al. 2001). As for acute stress, also chronic stress is able to alter many factors involved in the coagulation process. In particular, it was demonstrated an increase in fibrinogen and coagulation factors VII, VIII, and von Willebrand factor (Austin, Wissmann, and von Kanel 2013). In addition, other studies found that chronic stress is also able to increase the level of

Plasminogen-activator inhibitor-1 (PAI-1) and reduce t-PA (von Känel 2012). As regards the molecular mechanism by which stress is able to unbalance the coagulation system, it was found that the activation of the HPA-axis, inducing the release of catecholamines, stimulates β2-adrenergic receptors, in particular mediating their effects on endothelial cells. Only few minutes of stimulation are sufficient to activate the release of FVIII, active VWF and profibrinolytic t-PA from endothelial storage pools into the bloodstream (von Känel and Dimsdale 2000), to increase plasma levels of thrombin (von Känel et al. 2002) and also to induce the release of hepatic FVIII and affect the clearance of t-PA and D-dimer in the liver (Austin, Wissmann, and von Kanel 2013). In addition, it was found that sympathetic nerves hyper-stimulation in artery walls induced an increase in circulating t-PA (Hao et al. 2005).

The increased blood pressure induced by stress determines an efflux of plasma in the interstitial space of vessels thus concentrating non-diffusible large (i.e. >69 kDa) haemostatic molecules (Austin, Wissmann, and von Kanel 2013) that are in this way more in contact each other (Austin, Patterson, and von Känel 2011). Arithmetic adjustment for stress-haemoconcentration accounts for a portion of stress-induced haemostasis molecules as fibrinogen and VWF suggests that the intrinsic coagulation pathway is activated during stress (Austin et al. 2012).

Thrombotic risk after acute stress increases with age (Jern et al. 1989), and it was found that it is correlated also with a proportional increasing level of D-dimer (Wirtz et al. 2006). Regarding sex, the risk was found to be higher in men than in women after an acute event. It was showed that stress enhances the coagulation factor VIII only in male, while women have an increase in the activation of t-PA which promotes the fibrinolytic process (Jern et al. 1989).

Table 1. Changes in the levels of haemostatic factors after stress exposure.

↑ = increased level; ↓ = decreased level; - = no change. (Adapted from von Känel 2015).

Haemostatic factor	
Fibrinogen	↑
Factor XII	↑
Factor VII	↑
Factor VIII	↑
Von Willebrand factor antigen	↑
Thrombin-antithrombin complex	↑
Fibrin D-dimer	↑
Tissue-type activator antigen	↑
Tissue-type plasminogen activator activity	↑
Percent prothrombin time	↑
Plasminogen activator inhibitor-1	-/↑
Activated partial thromboplastin time	↓

2.1.6 Psychosocial stress and endothelial dysfunction

Endothelium represents a fundamental anatomic structure deputed to the control of vascular tone, angiogenesis, wound healing, smooth muscle cell proliferation, fibrosis, inflammation, and haemostatic balance. Classic risk factors for CVDs, altering the molecular pathways necessary to maintain the correct processes regulating the functions above mentioned, are at the bases of endothelial dysfunction (Widmer and Lerman 2014).

Of note, recently accumulating evidence showed that stress-related endothelial dysfunction could be an early risk factor predicting future development of CVDs.

Mental stress is able to directly enhance the release of pro-inflammatory cytokines and endothelin-1 leading to a lower release of nitric-oxide via the downregulation of endothelial nitric oxide synthase (eNOS) (Toda and Nakanishi-Toda 2011).

Human studies carried out with different paradigms of acute stress found that vascular resistance is enhanced and dilatation of the brachial artery is reduced after 30 and 90 minutes after the test (Ghiadoni et al. 2000, Sherwood et al. 1999), suggesting only a transient endothelial dysfunction. Interestingly it was reported that

intense emotions such as outbursts of anger and acutely depressed mood could increase by two times the risk of the acute coronary syndrome within two hours from the stressful triggering event (Mostofsky, Penner, and Mittleman 2014, Steptoe et al. 2006). However, it must be underlined that several studies failed to reach similar results for the heterogenicity of the responses to these paradigms of stress. In particular, it is hypothesized that this could be due to individual differences in the neuroendocrine coping mechanisms (Gerra et al. 2001) and the presence of people hyper-responsive to the stimulation of the sympathetic nervous system induced by stress (Rozanski, Blumenthal, and Kaplan 1999). Chronic stress disrupting hormonal homeostasis of glucocorticoids and catecholamines is able to induce not only metabolic abnormalities and inflammation but also endothelial dysfunction (Das and O'Keefe 2006). Studies on students with chronic sleep deprivation and under stress for examinations showed reduced vasodilation (Takase et al. 2004) and this parameter was even worst when in presence of sedentary lifestyle and smoking (Mancaş et al. 2008). Low socioeconomic levels in relationship with anxiety, depression, tension, hostility, and fatigue were found to determine impaired flow-mediated vasodilatation in both women and men and increase in carotid intima-media thickness only in men (Cooper et al. 2010, Mausbach et al. 2010, Chumaeva et al. 2010).

2.1.7 Psychosocial stress and the neurotrophin system

As already reported, chronic stress leads to an alteration in the structure of the central nervous system with a particular impact on the hippocampus region where it was shown that GCs continuous stimulation is at the basis of dendritic atrophy (Berton and Nestler 2006). Of note, it was observed that these alterations induced by stress are able to reduce the level of different neurotrophins in the same brain region and in the prefrontal cortex (Smith et al. 1995b, László et al. 2019).

Among them, the down-regulation of the Brain-Derived Neurotrophic Factor (BDNF) pathway was found to have an important molecular role in stress response. The response to stress, however, differs from region to region in the brain as demonstrated by the fact that its levels are reduced in the hippocampus but enhanced in the amygdala, suggesting a different involvement of these regions in neuronal structural plasticity (Lakshminarasimhan and Chattarji 2012). Interestingly, the administration of anti-depressant not only was able to counteract the effect of chronic stress but was also able to prevent BDNF reduction in the hippocampus and

protect neurons from atrophy (Chen et al. 2001). Following these and other results showing that symptoms of stress and pathologies related to it, such as depression, have in common a decrease in neurotrophic support and that infusion of these proteins (Shirayama et al. 2002) or gene overexpression (Govindarajan et al. 2006) leads to ameliorate pathological conditions, the “neurotrophic hypothesis” was formulated (Duman and Monteggia 2006). However, since rodent models with BDNF gene deletion do not show spontaneous depression phenotype, it could be hypothesized that there are other alternative pathways that working along with the neurotrophin are causative of the pathology suggesting that possible therapeutic strategies must take into consideration targets downstream BDNF pathway (Duman and Voleti 2012).

2.2 Psychosocial stress management

Since all the above describes evidence suggest that stress could be an important cause of psychological, inflammatory, metabolic and cardiovascular disease, different techniques for stress management were investigated. Among them, some techniques are devoted to improving physical health, such as exercise, while others are psychological interventions such as individual or group counseling or support of self-care.

2.2.1 Exercise

In the last few years, a growing body of the literature showed that psychological pathologies such as stress, anxiety, and depression might benefit from physical activity and exercise. In particular, it was shown that regular activity is associated with a better level of self-esteem and mood state and lower stress and anxiety levels (Anderson and Shivakumar 2013, DeBoer et al. 2012). The positive effect of exercise on mental disorders has been studied in depth founding that it is sustained by the integrative enhancement of the energetic and neuro-mediate homeostasis, by the modulation of the immune system, and by psychological improvements (Mikkelsen et al. 2017). In particular, it was observed that exercise activates mitochondrial function sustaining neuronal dendrites sprouting and axonal stability (Bansal and Kuhad 2016) and improving the performances of muscular fibers (Broskey et al. 2014). In addition, mitochondrial activity is also correlated to activation of thermogenesis that was found able to reduce the intensity of anxiety

episodes (Youngstedt et al. 1993). Exercise, stimulating the central nervous system, is able to enhance the endocannabinoid system (Heyman et al. 2012), to increase serotoninergic and adrenergic levels in the brain acting in the same way as antidepressant drugs (Wipfli et al. 2011), and to adjust the hormonal pathway of the HPA-axis which hyperactivation is known responsible for stress-related impairments (Salmon 2001). It also increases mTOR signaling in brain regions involved in cognition and emotional behavior (Lloyd et al. 2017). Besides this physiological effects, exercise might help to distract from negative thoughts especially when combined with music (Barwood et al. 2009) and increase self-esteem by generating a feeling of mastery which elevates mood (Middelkamp et al. 2017). Exercise affects inflammation system by reducing the number of total leukocytes (Apostolopoulos et al. 2016), by stimulating the production of anti-inflammatory cytokines such as TNF-alpha, IL-1, IL-8 and IL-15 in neurons, muscles and adipose tissue (Apostolopoulos et al. 2014, Schindler et al. 1990), by decreasing tool-like receptor (TLR) expression in monocytes (Gleeson 2007), and by enhancing the vagal tone thus having a positive impact in restraining the inflammatory cascade as well as reducing heart rate in chronic heart failure (Guiraud et al. 2013).

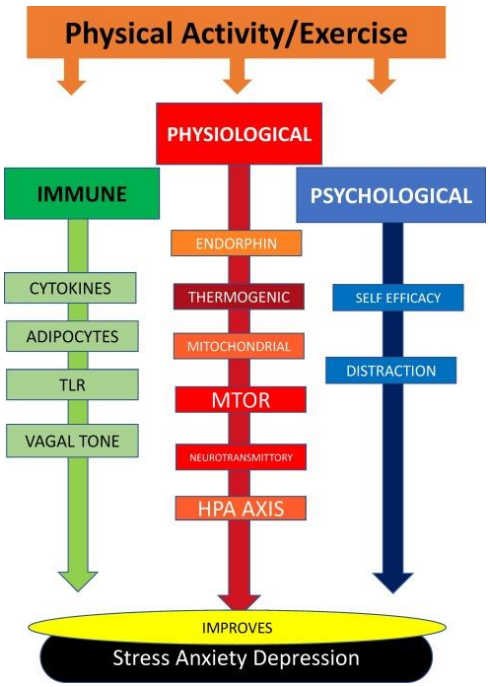


Figure 2. Representation of the systems mediating the beneficial effects of exercise on stress, anxiety, and depression.

TLR, toll-like receptors; mTOR, mammalian target of rapamycin signaling; HPA axis, hypothalamic-pituitary-adrenal axis (Mikkelsen et al. 2017).

2.2.2 Cognitive-behavioral therapies

Cognitive-behavioral therapies are based on the evidence that a better understanding of behaviours, emotions, and ideas could contribute to reducing distress and consequent anxiety or, in the worst cases, major depression (Giummarra et al. 2018, Shen et al. 2018). Different kinds of therapies was found to be effective in reducing psychological alteration. Psychodynamic therapy is based on the understanding of unconscious internal conflicts, interpersonal therapy is based on the understanding of the social interactions with family and friends and cognitive behavioural therapy (CBT) involves the understanding of negative thoughts and how they can lead to negative emotions. Other therapies combine relaxation, physical activity, and meditation such as deep breathing, progressive muscle relaxation, yoga, meditation, and mindfulness-based stress reduction. It must be underlined that in many cases psychotherapy show the same efficacy as antidepressant drugs and that these paradigms of interventions might have a fundamental role in the management of patients with early life stress, anxiety or depression issues (Farhang et al. 2019, Lloyd et al. 2018).

2.2.3 Pharmacological management

In line with the rules of interventions for psychosocial disorders, primary strategies for the treatment of stress must be conservative and aimed to promote the development of a personal strategy of response mechanisms from every patient. However, when despite this management stress leads to panic attacks traumatic recall associated with post-traumatic stress disorder (PTSD), and anxiety, drug therapy must be taken into account. Benzodiazepines, antidepressants, monoamine oxidase inhibitors (MAOIs), beta-adrenergic blocking agents and antihistamines are the class of drugs approved for the treatment of stress with different indications and timing of action (Curtis 2019).

3 The neurotrophin system

Neurotrophins are a protein family essential for the central nervous system development and maintenance promoting neural differentiation and survival, regulating axons and dendrites development, synaptogenesis and synaptic plasticity (McAllister, Katz, and Lo 1999, Huang and Reichardt 2001). Neurotrophin family includes four highly structurally correlated proteins: nerve growth factor

(NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-2 (NT-3) and neurotrophin-4/5 (NT-4/5). NGF was the first member to be identified (HAMBURGER and LEVI-MONTALCINI 1949). Only about thirty years later BDNF was isolated from the porcine brain (Barde, Edgar, and Thoenen 1982) and subsequently, the aminoacidic sequence was determined (Leibrock et al. 1989). The structural analogy between these proteins helped later to identify the other two members NT-3 and NT-4/5 in mammal's brains (Park and Poo 2013).

All the neurotrophins are synthesized as precursors proteins constituted by a pre-prodomain, a prodomain, and a mature domain. The pre-prodomain is cleaved and degraded after the neurotrophins have reached the Golgi apparatus let hypothesizing that its role is related to the correct shuttling from the endoplasmic reticulum. The resulting cleaved proteins are called proneurotrofins and have a weight of about 30-35kDa. Proneurotrophins then could undergo proteolytic cleavage or be directly secreted. Proteolytic cleavage determines the release of the mature domain of about 12-13kDa and the prodomain (often also referred to as propeptide) structure of about 15-17kDa (Seidah et al. 1996). Proteolytic cleavage is performed intracellularly by furins or pro-convertases (Seidah et al. 1996, Edwards et al. 1988), or extracellularly thanks to plasmin or different metalloproteases (Lee et al. 2001). Prodomain structures are highly conserved among the neurotrophins and also through the mammals, evolution, and mutation occurring in these regions are related to neurotrophins trafficking regulation from endoplasmic reticulum to cellular membrane during the release phase (Lee et al. 2001). Regarding the secretion of proneurotrophins, this process can follow two distinct pathways: regulated or constitutive. Regulated secretion is typical of the neurons (Cool et al. 1995) while smooth muscle cells, fibroblasts, and astrocytes secrete the proteins with constitutive secretion (Nielsen et al. 2001).

3.1 Receptors and signal transduction

The biological actions of neurotrophins described above are supported by the interaction of these proteins with two distinct classes of transmembrane receptors: Tyrosine-kinase receptors (Trk), including the forms TrkA, TrkB and TrkC, and neurotrophin receptor p75(NTR) a member of the superclass of Tumor Necrosis Factor Receptor (TNFR) (Huang and Reichardt 2003). Every single mature domain deriving from a different proneurotrophin binds with high affinity a specific receptor.

NGF binds to TrkA; BDNF and NT-4/5 bind to TrkB, while NT-3 binds preferentially TrkC but it could also bind TrkA and TrkB in specific cells (Huang and Reichardt 2003, Segal 2003). All neurotrophins are able to bind, even with low specificity, p75NTR which, in turn, is the specific receptor for every pro-neurotrophin (Lee et al. 2001).

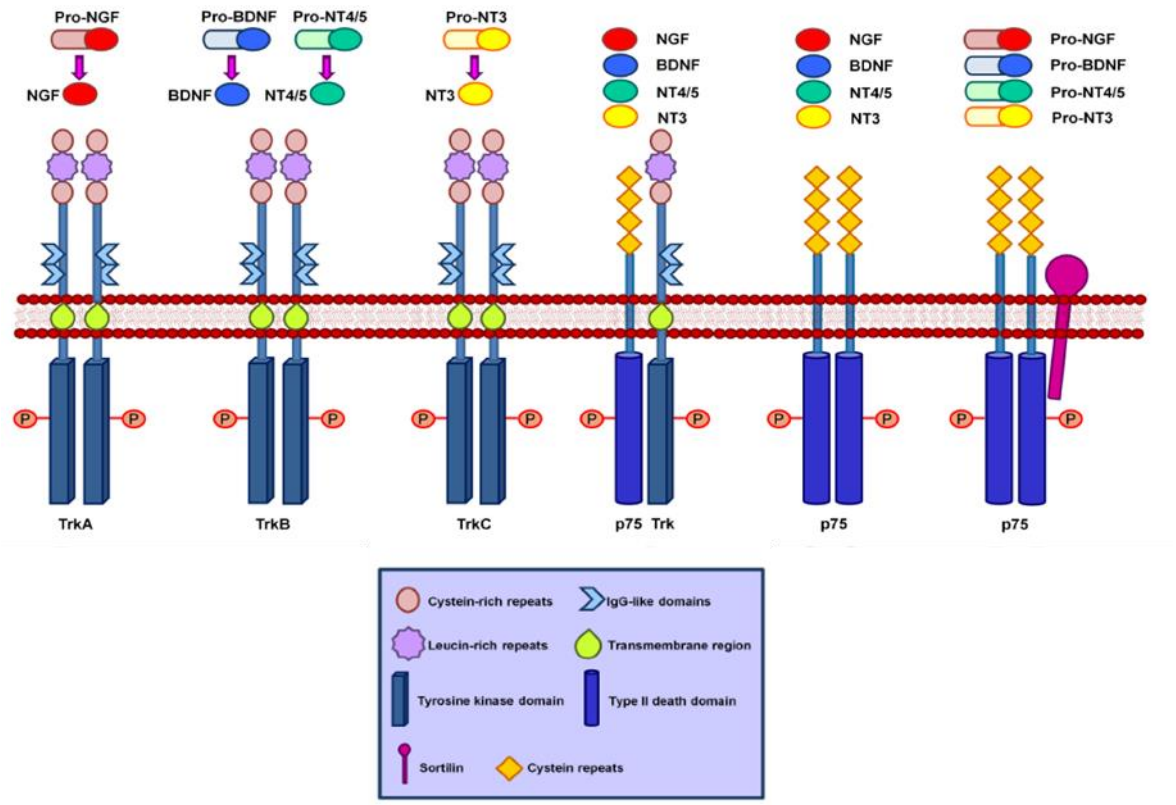


Figure 3. Receptors for the neurotrophins.

All the pro-neurotrophins bind to the p75NTR but not to Trk receptors. After proteolytic cleavage, every neurotrophin in its mature form binds with a specific Trk receptor but retains a weak ability to bind p75NTR (Bucci, Alifano, and Cogli 2014).

Trk receptors are constituted by a binding site containing multiple repetitions of leucine-rich-regions (LRR1-3), two cysteine clusters (C1 and C2) and two immunoglobulin-like domains (Ig1 and Ig2). They have a single conserved transmembrane domain followed by the tyrosine-kinase domain (Schneider and Schweiger 1991). The ligand specificity is determined by the structure of the Ig2 domain that couples with specific domains of every mature neurotrophin (Urfer et al. 1995, Urfer et al. 1998). The binding between the mature neurotrophin and Trk receptor determines the activation of three possible intracellular enzymatic pathways: phospholipase Cγ (PLCγ), phosphoinositide 3-kinase (PI3K) and

Ras/Raf/MEK/MAPK (Kaplan and Miller 2000). The binding of the neurotrophin with the Trk receptor determines its dimerization and the subsequent trans-phosphorylation of specific tyrosine residues (Y) at the cytosolic domain. It was shown that two specific phosphorylated tyrosine residues localized at the juxtamembrane domain and in the C-terminal (Y490 and Y785) are fundamental for the anchoring of adaptive molecules starting the different signal-transduction pathways (Stephens et al. 1994). Y490 phosphorylation determines the binding and phosphorylation of Shc, responsible for the recruiting of the complex Grb-2/SOS. This one leads to the activation of Ras, which activates transiently PI3K or the MAPK pathway. MAPK activation determines the phosphorylation of the transcriptional factor Camp Responsive Element Binding Protein (CREB). Complex Shc/Grb-2/Gab-1 or Ras activates PI3K promoting neuronal survival through the activation of protein kinase B (PKB/AKT) whose role is to inactivate the pro-apoptotic protein BAD.

Otherwise, the phosphorylation of Y785 determines the activation of PLCγ that hydrolyses phosphatidylinositol 4,5-bisphosphate (PI2P) to generate diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3), thus controlling intracellular Ca^{2+} levels and the activity of protein kinase C (PKC). This signaling seems important for maintaining synaptic plasticity (Gottschalk et al. 1999) and has a role in neurotrophin release from neurons (He, Gong, and Luo 2005). Interestingly, it was discovered that besides the normal structure of the TrkB receptor (also called full-length TrkB), there are also two splicing variants named TrkB-T1 (TrkB isoform Truncated-1), TrkB-T2 (TrkB isoform Truncated-2) and a novel one TrkB-T4 (TrkB isoform 4). All these three isoforms have the same extracellular and transmembrane domain as the full-length TrkB but lack the tyrosine-kinase domain which is substituted by respectively 23, 21 (Klein et al. 1990) and 83 amino acids (Forooghian et al. 2001) forming a short carboxyl-terminal tail. TrkB-T1 is highly expressed in the mature brain (Ohira, Shimizu, and Hayashi 1999) where it is known for its action of inhibiting the signal transduction mediated by full-length TrkB. TrkB-T1 forms and heterodimers with full-length TrkB preventing its phosphorylation and the subsequent signal transduction (Eide et al. 1996). It was also found that TrkB-T1 acts independently from full-length TrkB. It can directly bind and so sequestrate BDNF if the levels of this latter are too abundant in the inter-synaptic space and release it when they return physiological (Biffo et al. 1995). TrkB-T1 was also found

to be able to regulate the cytoskeleton structure in cultured astrocytes and glial cells as well as to control the Rho-GTPase activity (Fenner 2012). Also, TrkB-T2 is able to form heterodimers with full-length TrkB and blocking its signal transduction (Eide et al. 1996). However, its distribution in the brain is very poor (Stoilov, Castren, and Stamm 2002). TrkB-T4 was found for the first time in the kitten visual cortex (Forooghian et al. 2001) and subsequently, the mRNA was found also in human and mice brain, even if, as TrkB-T2, only in particular regions (Stoilov, Castren, and Stamm 2002). However, the lack of specific antibodies for TrkB-T2 and TrkB-T4 is now the major problem that researchers have to overcome to better understand these two isoforms.

As stated above, the p75NTR receptor has a high affinity for the proneurotrophins and mediates signal pathways that are mainly associated with neuronal apoptosis (Teng et al. 2005). It was found that to activate signal transduction, p75NTR has to coordinate with another protein called SorCS2 (Nykjaer et al. 2004, Teng et al. 2005). In particular, it was observed that the p75NTR receptor binds to the mature domain region while SorCS2 binds the prodomain region of the proneurotrophins (Teng et al. 2005, Anastasia et al. 2013). The ligand of the proneurotrophins with p75NTR determines the activation of several intracellular pathways such as stimulation of Jun-kinase and acid-sphingomyelinase, and the suppression of RhoA activity thus leading to apoptosis. On the other way, the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) mediates survival (Reichardt 2006). It was shown that TrkB activation not only mediates its own signals transduction but also is able to suppress Jun-Kinase cascade and sphingomyelinase hydrolysis but has no effect on the activation of the Nf-kB pathway (Dobrowsky, Jenkins, and Hannun 1995, Yoon et al. 1998). In this way, upon concomitant TrkB and p75NTR stimulation, the apoptotic signal mediated by p75NTR is suppressed while the activated Nf-Kb signal transduction contributes synergically to neuronal survival (Maggirwar et al. 1998). In these cases, it seems that the role of p75NTR is both to enhance the affinity of the neurotrophins for the TrkB receptor and concomitantly increase the pro-survival cascade (Bibel, Hoppe, and Barde 1999). Since it was observed that p75NTR expression is up-regulated in the presence of nervous system lesions it was hypothesized that the main role of its stimulation is to help eliminate damaged cells. (Beattie et al. 2002).

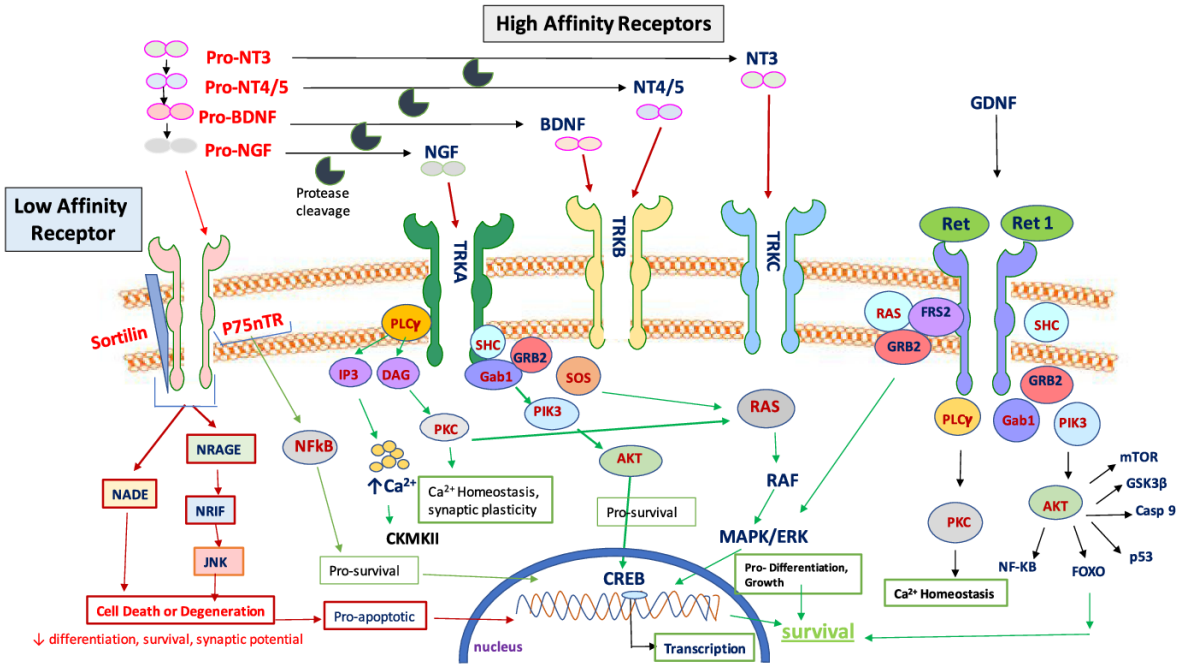


Figure 4. Signal transduction pathways of the neurotrophins.

Trk receptors mediate the three signal transduction pathways: phospholipase Cγ (PLCγ), phosphoinositide 3-kinase (PI3K) and Ras/Raf/MEK/MAPK. The binding of the neurotrophins to the p75NTR mediates the activation of the NF-κB and the Jun kinase, and the regulation of Rho activity (Kashyap et al. 2018).

3.2 Brain-Derived Neurotrophic Factor (BDNF)

3.2.1 BDNF gene

In humans, the BDNF gene is located on chromosome 11, it has a length of about 70kb and is constituted by eleven exons (named I-IX plus the exons Vh and VIIIh). Notably, exon IX is the only one coding for the pre-proBDNF protein and in this exon is located the single acceptor site for transcripts splicing (Pruunsild et al. 2007)

It was observed that there are eight distinct classes of transcripts. Transcription can start from exon I, II, III, IV, V, Vh, VI, VII and the splicing donor site of every one of these exons are linked at the acceptor site on exon IX. However, recently other splicing donor sites that lead to the generation of 5' UTR regions were identified at exons II, VI and VII (Pruunsild et al. 2007). Transcription starts from the ATG sequence in exon IX, however, this sequence was also identified in exons I, VII and VIII let hypothesize the possibility to generate different BDNF transcripts with a longer N-terminal sequence (Pruunsild et al. 2007). The exon IX, coding for the protein and the 3'UTR region, is subjected to internal splicing and has two alternative polyadenylation sites leading to two distinct mRNA populations

characterized by different lengths of the 3'UTR regions (Timmusk et al. 1993, An et al. 2008).

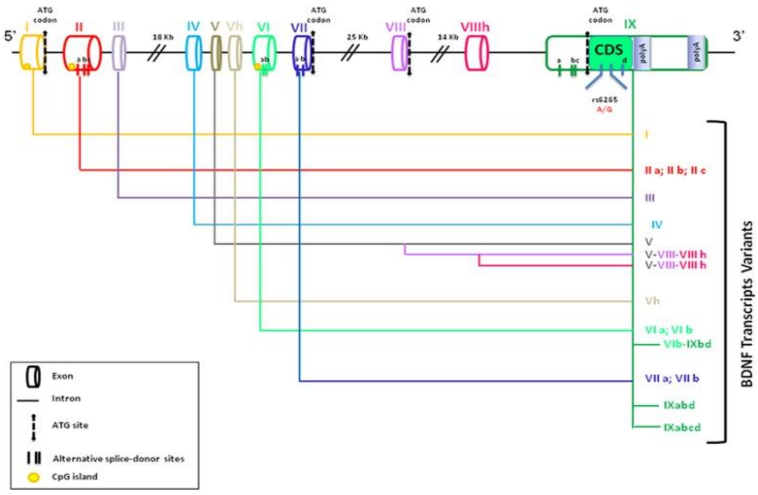


Figure 5. Structure of the human BDNF gene.

The human BDNF gene contains 11 exons (I-IX plus Vh and VIIIh), leading to the formation of different transcripts. The coding sequence for the BDNF protein is located at exon IX (Cattaneo et al. 2016).

3.2.2 BDNF synthesis and secretion

As all the neurotrophins, the BDNF is synthesized in the endoplasmic reticulum as a pre-proneurotrophin named pre-proBDNF. Pre-proBDNF is constituted by the pre-domain, the pro-domain and the mature domain. Thanks to the pre-domain signal sequence, the immature form of the protein are carried to the Golgi apparatus where the proteolytic cleavage between the pre-domain and the pro-domain takes place, generating the proBDNF protein (Lu 2003, Foltran and Diaz 2016). This point, two regions of the prodomain, named box2 and box3, interact with the protein Sortilin, located intracellularly the Golgi apparatus that helps the correct folding of the proBDNF (Chen et al. 2005). Subsequently, proBDNF could undergo another proteolytic cleavage between the pro-domain and the mature domain, either at the intracellular or extracellular level. However, a certain amount of the protein could be secreted as proBDNF without undergoing any other proteolytic process (Foltran and Diaz 2016, Mizui et al. 2016). It was observed that the amount of proBDNF undergoing proteolytic cleavage or being release as it changes during different stages of brain maturation. During the post-natal phase, the majority of the protein is released as proBDNF, while after the complete maturation mature BDNF is the dominant one (Yang et al. 2014). Intracellular cleavage could take place or in the

Golgi apparatus thanks to the enzyme Furin or in the secretory vesicles of both the constitutive or regulated secretion pathway, thanks to different enzymes of the family of proconvertases (Lu, Pang, and Woo 2005). Extracellularly the proteolytic cleavage is mediated by Plasmin or different Metalloproteases enzymes (Pang et al. 2004).

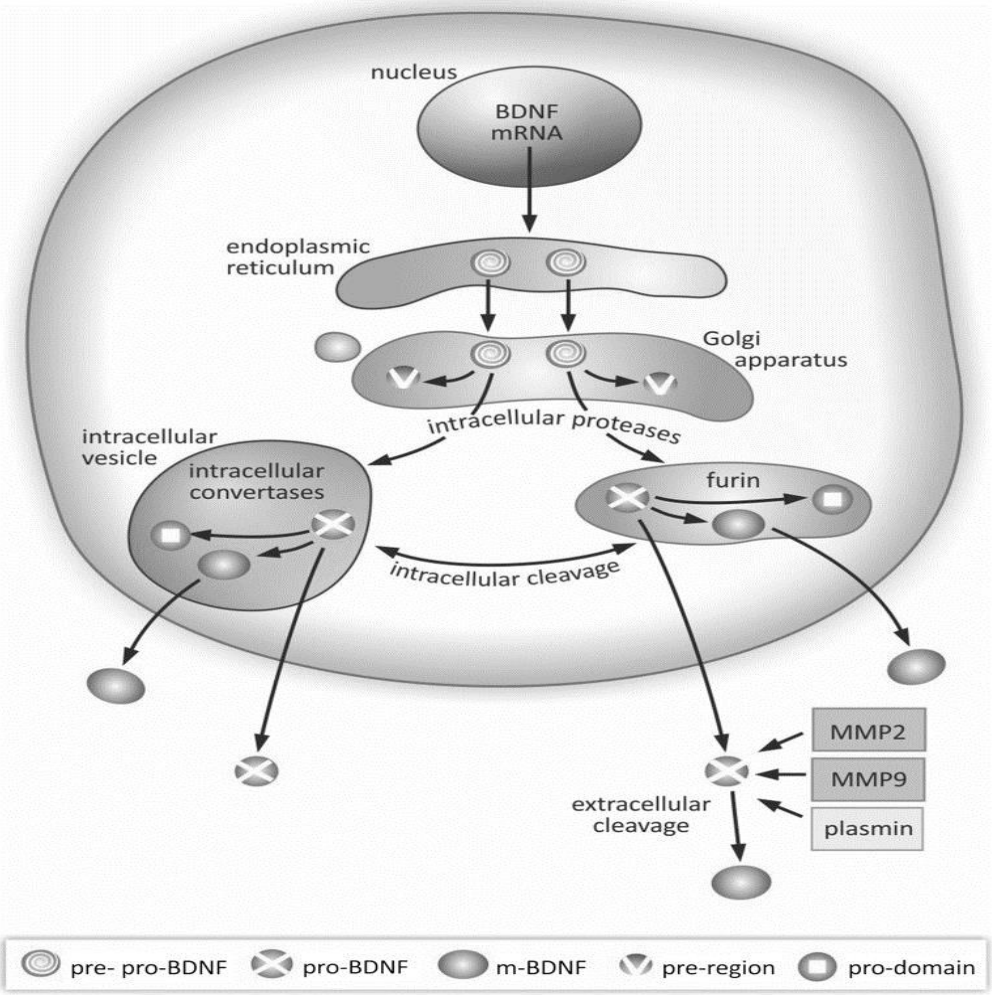


Figure 6. Schematic representation of the synthesis and maturation of BDNF.
BDNF mRNA is translated in the endoplasmic to its Pre-ProBDNF protein form. Then the protein is shuttled to the Golgi apparatus and in intracellular vesicles where it is cleaved to its ProBDNF or mature BDNF isoforms and then addressed to the secretion pathways (Kowiański et al. 2018).

Once the proBDNF protein is correctly folded, it could be addressed either to the constitutive or to the regulated secretion pathway. In the constitutive secretion pathway, ProBDNF and cleaved mature BDNF are loaded in small secretory granules that, fusing with the cellular membrane of the cell, release their content without any activation mechanism needed. This mechanism is typical of non-neuronal cells. In neurons and cultured astrocytes, it was observed that proBDNF

and mature BDNF trafficking is mediated by bigger vehicles that run along the axons and are released at the dendrites after Ca^{2+} mediated depolarization (Mowla et al. 2001). It was observed that in the mature domain of BDNF there are 4 amino acids, Ile 16, Glu 18, Ile 105 and Asp 106, which are fundamental for the regulated secretion pathway. The substitution of one of them with a different amino acid, for example, an Alanine, determines a reduction in the regulated secretion and an increase of uncontrolled constitutive release with a deleterious effect on neuron network (Lou et al. 2005). The above-mentioned amino acid residues interact in the vesicles with basic amino acid residues of the protein Carboxypeptidase E (CPE), the receptor responsible for proBDNF and matureBDNF sorting through the regulated secretion (Cool et al. 1995).

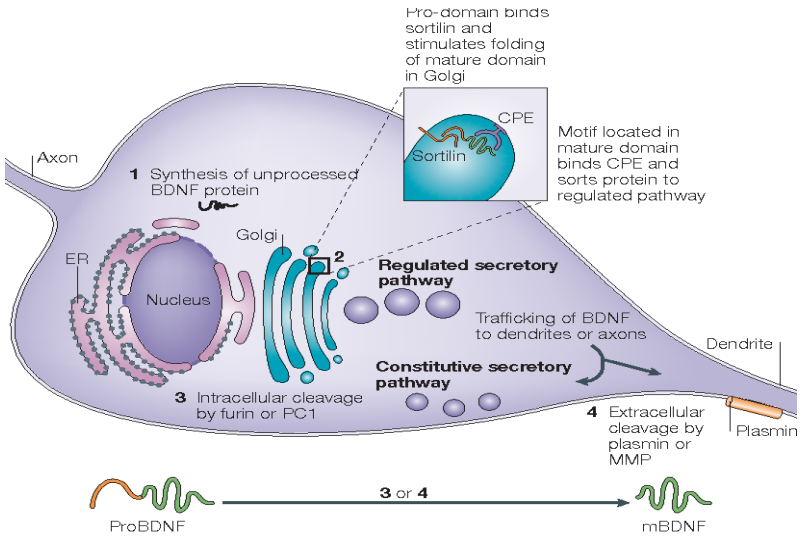


Figure 7. Schematic representation of the BDNF secretion via the regulated and constitutive pathways. In the constitutive secretion pathway, BDNF and ProBDNF are stored in small secretory granules and are release directly, without any activation mechanism. In the regulated secretion pathway, the BDNF prodomain binds to the sortilin, which is responsible for its correct folding. The mature domain of BDNF interacts with the carboxypeptidase E (CPE) and it is subsequently addressed to the site of release (Lu, Pang, and Woo 2005).

3.2.3 Central and peripheral functions of BDNF

During development, BDNF is fundamental for the differentiation and survival processes of the central and peripheral nervous system contributing to axons growth and dendritic morphology. In the terminal phases of development and in the developed system, BDNF is essential to maintain synaptic plasticity and the correct neuronal transmission (Bibel and Barde 2000, Binder and Scharfman 2004). BDNF

is found in all the cerebral areas, in particular, its protein levels are higher in the hippocampus, amygdala, cerebral cortex and hypothalamus (Katoh-Semba et al. 1997). Several studies showed that alteration in the BDNF level is associated with the onset and progression of neurodegenerative and neuropsychiatric disorders such as Parkinson's', epilepsy, psychosis, anxiety, and depression (Nagahara and Tuszynski 2011, Lu et al. 2013). Focusing on major depression, it was observed that in autoptic samples of depressed patients, the protein level in the prefrontal cortex and hippocampus are reduced if compared to controls (Dwivedi et al. 2003) and that BDNF level is increased after antidepressant treatment (Chen et al. 2001). Regarding stress, studies on animal models and patients clearly showed that this condition is associated with a reduction of BDNF, in particular in the hippocampus and in the medial prefrontal cortex where BDNF mediates structural and neurotrophic support to neurons (Duman 2004) (Smith et al. 1995a). Stressful conditions, hyper activating the HPA-axis and enhancing cortisol release in the brain can mediate alterations in synaptic plasticity and neurogenesis (Duman and Monteggia 2006, McEwen 2007) and it was shown that cortisol over-stimulation is related to a reduction in glucocorticoid receptors and associated with lower BDNF synthesis and a reduction in the hippocampal volume (Duman 2004, Smith et al. 1995a).

Even if BDNF mRNA and protein level is detected at a high level in the central and peripheral nervous system, it must be underlined that it could also be found in peripheral organs such as liver, thymus, heart, lung, and spleen (Ernfors et al. 1990, Maisonpierre et al. 1990, Maisonpierre et al. 1991, Katoh-Semba et al. 1997). BDNF plays a role in angiogenesis, both in the early stages of heart development and in tumor-related one (Pearse et al. 2005, Yang et al. 2006). It was found that BDNF plays a critical role during cardiovascular system development (Caporali and Emanueli 2009). The expression of BDNF and its receptor TrkB is detectable from the first phases of fetal development (Kermani and Hempstead 2007) and it was observed that lacking BDNF is associated to endothelial cells apoptosis and reduction of cell-to-cell contact in developing cardiac vessels, leading to reduced contractility, haemorrhage and perinatal death (Donovan et al. 2000). In addition, BDNF and TrkB are essential in the adult cardiovascular system and are directly involved in the neovascularization that is observed after heart ischemia (Kermani et al. 2005). Angiogenesis could be mediated by two distinct but connected

mechanism. BDNF can directly stimulate the endothelial cells present in the vessel and expressing TrkB thus contributing to their survival or, alternatively, can act as a chemotactic factor enhancing the mobilization and recruitment of myeloid cells expressing TrkB receptor (Kermani et al. 2005, Kermani and Hempstead 2007).

Of note, blood circulating cells such as lymphocytes and monocytes are able to produce BDNF (Edling et al. 2004, Kerschensteiner et al. 1999, Schulte-Herbrüggen et al. 2005) and it is reported that self-produced BDNF is essential for eosinophils to survive and sustain the allergic response (Nockher and Renz 2005, Raap et al. 2005). BDNF can be detected in both plasma and serum but in different concentrations. Serum BDNF levels are 20 to 100-fold the ones found in plasma and this can be explained by the fact that BDNF is released from platelets (Fujimura et al. 2002) and leukocytes (Tuck et al. 2009) during the clotting process (Amadio, Sandrini, et al. 2017). BDNF is present in platelets and can be found in two distinct pools: cytoplasmic and stored in α-granules representing respectively 70 and 30% of the total content. Of note, it was demonstrated that after platelet activation by PAR1, only the BDNF contained in α-granules is secreted (Fujimura et al. 2002) along with other factors contained only in these reservoirs such as VEGF (Italiano et al. 2008). Different studies tried to understand the origin of platelet BDNF.

Tamura et al showed that BDNF is expressed in human megakaryoblastic leukaemia MEG-01 cell line only after stimulation with TPO and cytokines (Tamura et al. 2012), and one of the most recent publication definitely provide evidence that BDNF is expressed in human and rat megakaryocytes while it is not in mice ones (Chacón-Fernández et al. 2016), supporting the theory that BDNF platelets derived from their progenitors cells, the megakaryocytes.

However, platelets can also take-up BDNF from the environment through a mechanism not identified yet (Serra-Millàs 2016). Understanding the origin of platelet BDNF could be important in order to clarify the biological meaning of plasma and serum BDNF which levels have been often associated with different pathologies. Low level of plasma BDNF has been correlated with an increased risk to develop myocardial infarction (Manni et al. 2005), and represent an independent predictor of 4-year coronary and all-cause mortality (Jiang et al. 2011). Similarly, low serum BDNF was been associated with the increased risk of CVD and mortality (Kaess et al. 2015). Interestingly, a higher level of serum BDNF was found in the blood samples of patients with unstable angina versus the ones with stable angina

and this level positively correlates with soluble P-selectin (Lorgis et al. 2009, Ejiri et al. 2005), supporting the link between circulating BDNF and platelet activation.

Moreover, BDNF regulates many functions of the immunity system both in the nervous system and in peripheral tissues. It was shown that after physical lesions, neurodegeneration or infective and autoimmune diseases in the nervous system, activated T and B lymphocytes as well as oligodendrocytes release an important amount of BDNF contributing to neuroprotection and reducing neuronal damage (Kerschensteiner et al. 1999). In peripheral tissues, the BDNF released from the cells of the immune system was shown to have an immunomodulatory effect (Asami et al. 2006).

BDNF released from macrophages stimulates in an autocrine way their phagocytic activity and promoting IL-11β secretion through the activation of full length and truncated TrkB receptors (Asami et al. 2006). Moreover, it seems that BDNF could play an important role in the onset and progression of atherosclerotic plaque vulnerability. In animal models, BDNF and its receptor TrkB were shown to be highly expressed in atherosclerotic coronaries and this is associated with enhanced infiltration of mononucleated cells, activation of metalloproteases and stimulation of PAI-1 production by endothelial cells (Pepper 2001, Sun et al. 2006). In pathognomonic atherosclerotic coronary arteries, BDNF was found to co-localize with macrophages and smooth muscle cells and within cells of intima and adventitia (Ejiri et al. 2005). It seems that high levels of BDNF could contribute to the onset and progression of the atherosclerotic lesion through a mechanism of oxidative injury since it was shown that in vitro treatment of smooth muscle cells with BDNF is able to enhance their oxidative stress (Lorgis et al. 2009).

In addition, BDNF has a role in the regulation of the energetic homeostasis of the organism and alterations are related to metabolic dysfunction.

It was demonstrated that TrkB receptors are abundantly expressed in the hypothalamus and in the dorsal vagal complex that are the two main brain areas associated with the regulation of food intake and energy homeostasis (Conner et al. 1997, Yan et al. 1997). BDNF, stimulating TrkB receptors and acting as an effector of the melanocortin signal pathway in these areas is able to exert its anorectic function (Bariohay et al. 2005) (Lebrun et al. 2006). This role of BDNF was understood thanks to genetically modified animal models in which heterozygous deletion of the BDNF gene (Kernie, Liebl, and Parada 2000), and reduction of the

expression of TrkB (Xu, Goulding, et al. 2003) were associated with higher food intake and obesity. These data were confirmed in humans where 58% of children carriers deletion of chromosome 11p have heterozygous BDNF deletions and higher Body Mass Index (BMI). By 10 years of age, 100% of the patients with BDNF deletion were obese (Han et al. 2008). Moreover, Genome-Wide Association Studies (GWAS) showed a clear association between variants in genetic *loci* close to BDNF gene and obesity, body weight and body mass index (BMI) (Thorleifsson et al. 2009, Speliotes et al. 2010).

Starting from this evidence, different research groups tried to identify the role of BDNF in the adipose tissue. Firstly, it was demonstrated that BDNF levels decrease gradually during adipogenesis (Cheung et al. 2007, Bernhard et al. 2013) and that it is silencing in pre-adipocytes leads to a reduction in the differentiation ability (Bernhard et al. 2013). Interestingly, Adipoq-BDNF conditional knock-out mice show no difference in BDNF protein content in the adipose tissue when compared to WT littermates, suggesting that mature adipocytes do not contribute to BDNF production. On the contrary, Fabp4-BDNF conditional knock-out, determining a deletion not only in adipocytes but also in other cell types such as afferent nerve fibers and macrophages (Fu, Luo, and Lopes-Virella 2000, Martens, Bottelbergs, and Baes 2010), has low BDNF levels (Nakagomi et al. 2015). These studied provide evidence that the major sources of BDNF in adipose tissue are represented by macrophages, pre-adipocytes and other stromal-vascular components (Barouch et al. 2001, Nakagomi et al. 2015).

Overall, these data clearly show an important role of BDNF in non-neuronal physiology and pathology, thus making new efforts necessary for the comprehension of its mechanisms of action.

3.2.4 The BDNF Val66Met polymorphism

BDNF gene is found to be subject to several mutations of its sequence, most of which are single-nucleotide polymorphisms (SNPs). Among them, the most characterized is the one known as rs6265, which involves a guanine to adenine (G>A) substitution at nucleotide 196 (G196A). This single nucleotide substitution leads, during the translation process, to the insertion of a methionine instead of valine in position 66 (Val66Met) of the prodomain of BDNF protein (Egan et al. 2003). This polymorphism is present only in humans and its allelic frequency is 20-

30% among the Caucasian population (Hashimoto, Shimizu, and Iyo 2004) and up to 40-50% among the Asian population (Choi et al. 2006). If the heterozygous form of the SNP per se is not associated with pathological conditions, the homozygous one is associated to reduction of hippocampal volume and consequent poor performance on hippocampal-dependent memory tasks (Egan et al. 2003) and higher susceptibility to develop neuropsychiatric disorders such as Alzheimer's disease (Ventriglia et al. 2002) Parkinson's disease (Momose et al. 2002), schizophrenia (Neves-Pereira et al. 2005, Rosa et al. 2006), schizoaffective disorder (Lencz et al. 2009), bipolar disorder (Sklar et al. 2002, Müller et al. 2006), anxiety and major depression (Hosang et al. 2014, Zhao et al. 2018). The molecular mechanism(s) by which the mutation leads to this phenotype is not totally clarified yet. The most accepted hypothesis is that this is the consequence of a reduction in the regulated secretion of the mature form of the protein due to the fact that the mutation in the prodomain region alters the interaction with the protein Sortilin involved in BDNF maturation (Chen et al. 2004, Chen et al. 2005). At the moment, no crystallized structures of BDNF protein containing the Val66Met mutation and the surrounding amino acidic residues are available. However, a recent *in silico* analysis showed that the mutated protein has no difference in flexibility and surface-to-volume ratio while it affects essential motions, hydrogen-bonding and secondary structure particularly at its pre and pro-domain supporting the hypothesis that the mutation has a role in BDNF folding and maturation (De Oliveira et al. 2019).

Interestingly, it was also observed that the prodomain could be actively secreted by neurons and that in presence of the Val66Met polymorphism it can act as an independent ligand for the receptor SorCS2 which, coupling with p75NTR, is able to inhibit axonal growth (Anastasia et al. 2013). As already reported, BDNF can be measured in plasma or serum and different studies were carried out to understand if the Val66Met polymorphism is accompanied by a different level of circulating BDNF. Interestingly, in healthy subjects, this polymorphism was associated with higher levels of mature BDNF in serum (Lang et al. 2009), while no differences were found in plasma BDNF l (Tramontina et al. 2007, Karege et al. 2005, Terracciano et al. 2010). Reduced BDNF levels were detected in patients with depression (Bocchio-Chiavetto et al. 2010), while no information is available about the levels of BDNF in cardiovascular patients carrying Val66Met polymorphism. However, these results must be analyzed in a critical way since there are no standardized

procedures accepted overall and that different articles showed how the measurement of BDNF level in plasma and serum could be subjected to several biases due to the method of sample collection and manipulation and the time and temperature of storage (Amadio, Sandrini, et al. 2017, Zuccato et al. 2011).

As well as for other polymorphism correlated with mood disorders (Bondy 2007), also the Val66Met SNP was found to be associated with cardiovascular disease. In particular, it was shown that homozygous carriers of the Met with concomitant history of major depressive disorder have a higher risk to develop coronary artery disease (Bozzini et al. 2009, Liu et al. 2014). In addition, it was recently demonstrated that mice carrying the polymorphism in homozygosis present a pro-thrombotic phenotype related to platelet hyperactivation, altered coagulation system and enhancement of proteins involved in inflammation and thrombosis (Amadio, Colombo, et al. 2017). Of note, also in a cohort of patients the presence of the polymorphism in homozygosis is associated with a higher propensity for arterial thrombosis related to acute myocardial infarction (Amadio, Colombo, et al. 2017).

On the contrary, Jiang et al. demonstrated that the BDNF Met/Met genotype has a protective effect on the occurrence of unstable angina pectoris (Jiang et al. 2009). In line with this data, the CATHGEN study at Duke University Hospital showed that the Val/Val genotype was associated with a higher risk than Met carriers for clinical CVD events, with greater odds of having more diseased vessels, and lower left ventricular ejection fraction (Jiang et al. 2017).

As already reported, the alteration in the BDNF signal pathway is associated with HPA-axis dysfunctions. In line with this, it was found that an animal model carrying the Val66Met polymorphism shows a reduction in cortisol response when compared to wild-type after a standardized stress protocol (Alexander et al. 2010).

2. AIM OF THE STUDY

1 Aim of the study

Despite the huge growth in knowledge and advances in the prevention and treatment of cardiovascular disease (CVD), this pathology is still the leading cause of morbidity and mortality in the world, reaching 23.3 million by 2030 (Mathers and Loncar 2006). It was recently reported that canonical risk factors for CVD are predictors of about 75% of total morbidity and mortality, raising the awareness on the need to identify new dynamics involved in the onset and progression of these pathologies (Strike and Steptoe 2004). Interestingly, environmental factors such as stress, depression, and anxiety were recently included as new risk factors for CVD. Indeed, it was demonstrated that they may not only modulate the onset and progression of CVD but also influence the response to pharmacological treatments (Cohen, Edmondson, and Kronish 2015). This evidence made necessary to raise the awareness in clinical practice and to coin the term "behavioral cardiology" in which importance should be given to *"the recognition that adverse lifestyle behaviors, emotional factors, and chronic life stress can all promote atherosclerosis and adverse cardiac events"* (Rozanski et al. 2005). In particular, the role of the new discipline would be a deeper understanding of the pathophysiology of behavior-related CVDs and the development of effective therapeutic interventions both for modifying high-risk lifestyles and behaviors and for reducing psychosocial risk factors for patients. Besides the canonical pharmacological intervention, a growing body of literature showed that psychological pathologies might benefit from physical activity and exercise. In particular, it was shown that regular activity is associated with a better level of self-esteem and mood state and lower stress and anxiety levels (Anderson and Shivakumar 2013, DeBoer et al. 2012). In addition, the 2016 European Guidelines on CVD prevention in clinical practice (Piepoli et al. 2016), strongly recommended regular physical exercise (PE) as management for the prevention and treatment of CVD both in healthy people and patients with metabolic disorders. Regular PE was found to be able to reduce systemic inflammation, improve endothelial function, decrease platelet and leukocyte activation and halt the progression of coronary stenosis (Bruunsgaard 2005, Ertek and Cicero 2012, Schuler, Adams, and Goto 2013, Winzer, Woitek, and Linke 2018, You et al. 2013). In this context, the common human BDNF Val66Met variant through the reduction of the activity-dependent secretion and signaling of mature BDNF, is associated not only to neuropsychiatric disorders, anxiety and a higher susceptibility to stress (Tsai

2018), but was also found to contribute to the individual propensity for arterial thrombosis related to AMI in a human cohort (Amadio, Colombo, et al. 2017) and to eating disorders and obesity (Beckers et al. 2008, Wu et al. 2010, Xi et al. 2013, Zhang et al. 2014, Zhao et al. 2014). Interestingly, a knock-in mouse carrying the human BDNF Val66Met polymorphism well recapitulate all these disorders observed in human patients showing a depression-like/anxiety related-behavior and a significantly higher body weight than wild-type littermates (Chen et al. 2006), associated with a pro-inflammatory and pro-thrombotic phenotype (Amadio, Colombo, et al. 2017). Interestingly, stressful conditions unveil the anxious/depressive-like behavioral phenotype in heterozygous BDNFVal66Met ($BDNF^{Val/Met}$) mice. In addition, it was found that in homozygous Met mice the beneficial effect of exercise on neurobiological changes is impaired. These data suggest an important involvement of Met allelic mutation in terms of gene-environment interaction (GxE) regarding the behavioral profile. However, no data are available regarding these GxE interactions in terms of cardiovascular risk.

Starting from this evidence, the aim of this study was to highlight the impact of the interplay between Met allele environmental factors on the risk of arterial thrombosis. To reach this goal, homozygous ($BDNF^{Met/Met}$) and heterozygous ($BDNF^{Val/Met}$) mutant mice carrying the BDNFVal66Met mutation underwent spontaneous physical exercise or restraint stress protocols respectively as means to evaluate positive or negative environmental factors.

Homozygous BDNFMet/Met mice were used to evaluate the possible positive effect of physical exercise in relationship to Met allele since it is demonstrated that this animal model *per se* displays a higher body weight and a pro-thrombotic phenotype.

Heterozygous BDNFVal/Met mice were used to evaluate the possible negative effect of chronic stress in relation to the presence of the Met allele since it is demonstrated that this model does not show *per se* a pro-thrombotic phenotype.

Attention was given to understand the cellular mechanism(s) at the bases of the changes observed.

In particular:

1) to investigated the positive effect of life-style in preventing the propensity of thrombosis, homozygous $BDNF^{Met/Met}$ mice were exposed for four weeks to voluntary physical exercise and adipose tissue profile and pro-thrombotic phenotype has been evaluated

2) to assess whether stressful conditions unveil the prothrombotic phenotype in heterozygous BDNF$^{Val/Met}$ mice were exposed for seven days to sub-chronic stress and platelet activation, bone-marrow megakaryocyte profile, as well as gene expression of the arterial vessel wall, was analysed .

3. RESULTS I

1 Characterization of the white adipose tissue depots in BDNF$^{Met/Met}$ mice.

As previously reported in the literature, we observed that BDNF$^{Met/Met}$ mice have a significantly higher body weight when compared to BDNF$^{Val/Val}$ (34.75 ± 2.56 g. vs 31.25 ± 1.15 g, $p < 0.01$) (Figure 1A). Of note, the percentage of both inguinal white adipose tissue (ingWAT) and epididymal white adipose tissue (epiWAT) on total body weight were significantly greater in BDNF$^{Met/Met}$ mice compared to BDNF$^{Val/Val}$ (Figure 1B and 1C).

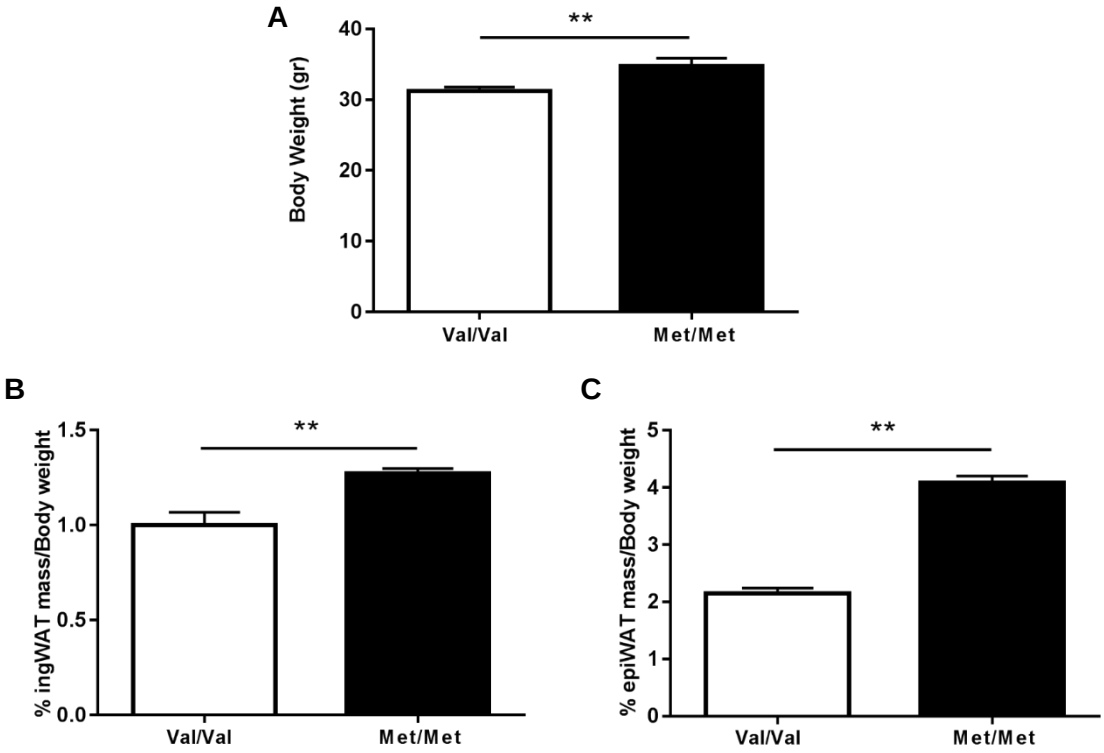


Figure 1. Weight of white adipose tissue depots in BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. (A) Bodyweight, percentage of (B) inguinal (ingWAT) and (C) epididymal (epiWAT) white adipose tissue on total mouse body weight. (i) Data are expressed as mean ± SEM. n = 6 mice/group. Student's t-test. ** $p < 0.01$.

Adipose tissue depots were analysed by histological examination, showing no difference in the frequency distribution of adipocyte sizes in ingWAT, while in the epiWAT the BDNF$^{Met/Met}$ mice showed enrichment in small-size and a reduction in middle-size adipocytes when compared to BDNF$^{Val/Val}$ (Figure 2).

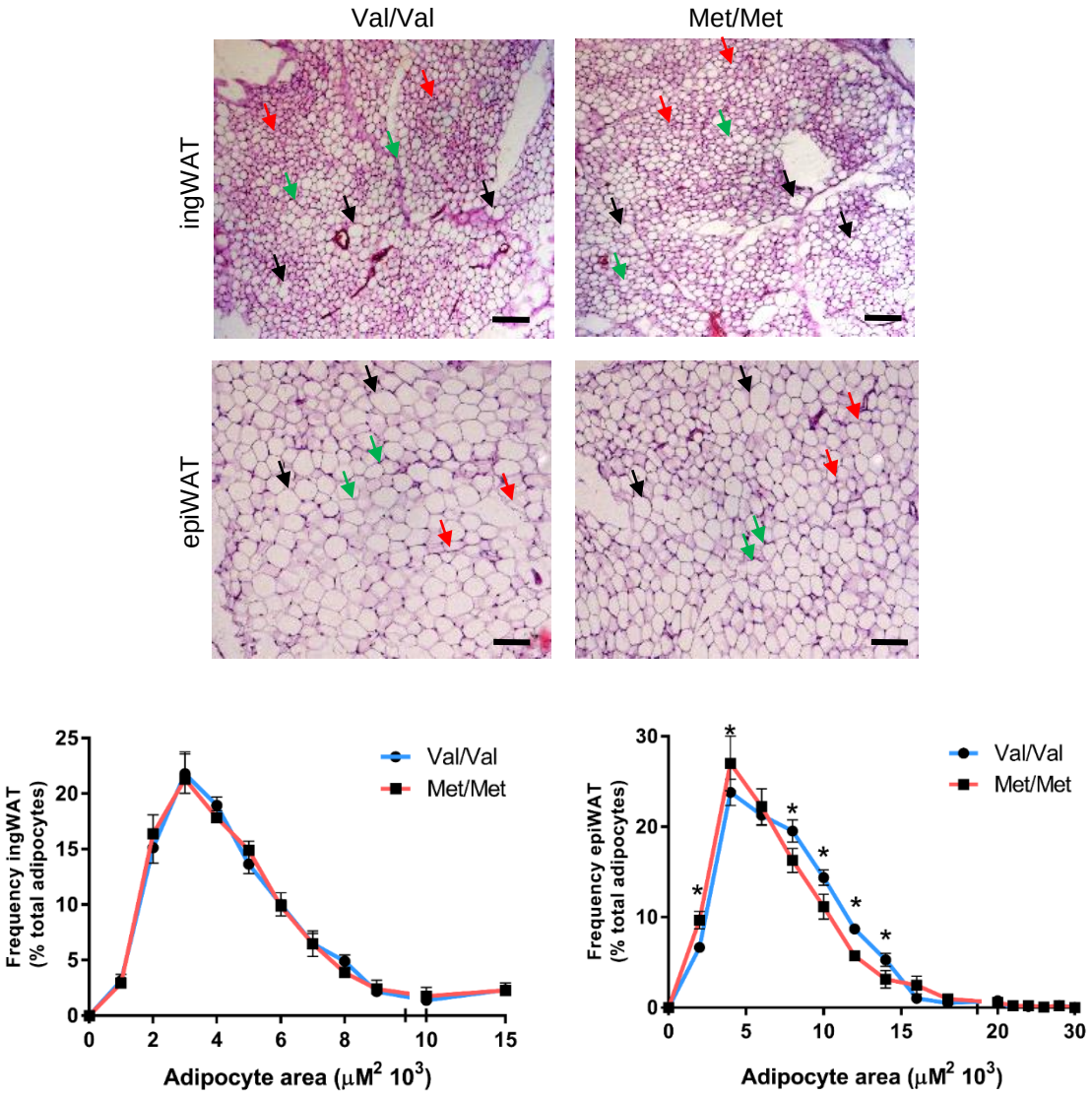


Figure 2. Representative images and adipocytes size distribution of white adipose tissue depots in BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. Representative hematoxylin and eosin (H&E) staining images and analysis of the frequency distribution of adipocyte sizes in ingWAT and epiWAT. Size bar: 100 μm. Black arrow: large adipocytes, green arrow: medium adipocytes and red arrow: small adipocytes. Data are expressed as mean ± SEM. n = 6 mice/group. Student's t-test. * $p < 0.05$,

Then the molecular signature underlying the distinct morphological feature of the epiWAT has been investigated. The expression of Pparγ, C/ebp-α and C/ebp-β genes, key regulators of the adipogenic program, along with Adipoq are reduced in BDNF$^{Met/Met}$ than BDNF$^{Val/Val}$ mice, while no differences were found regarding the expression of Fabp4 (Figure 3A). BDNF Val66Met polymorphism was also associated with an increase in the mRNA level of Adra2a, Sirt1, and Sorl1, genes

known to regulate energy balance and adipocyte morphology (Figure 3A, 3B and 3C).

The epiWAT of BDNF$^{Met/Met}$ mice displayed a pro-inflammatory profile with increased expression levels of Il-6, Tnf-α, Tgf-β, Mcp-1, and Pai-1 when compared to BDNF$^{Val/Val}$, although a similar mRNA level of TF was found (Figure 3B).

Concomitantly with the enhanced inflammatory profile, epiWAT of BDNF$^{Met/Met}$ was accompanied by greater expression of CD80, an M1 inflammatory macrophage marker, and with a reduction of CD163, an alternatively activated M2 macrophage marker (Figure 3B).

Of note, BDNF$^{Met/Met}$ mice have a higher BDNF mRNA level in epiWAT. Interestingly, epiWAT expresses the TrkB-full length and the TrkB-T1 of the BDNF receptors while no mRNA for the Trk-T2 truncated isoform was detected. no differences were found between the genotypes. The expression of TrkB-full length and the TrkB-T1 did not differ between the two genotypes (Figure 3C)

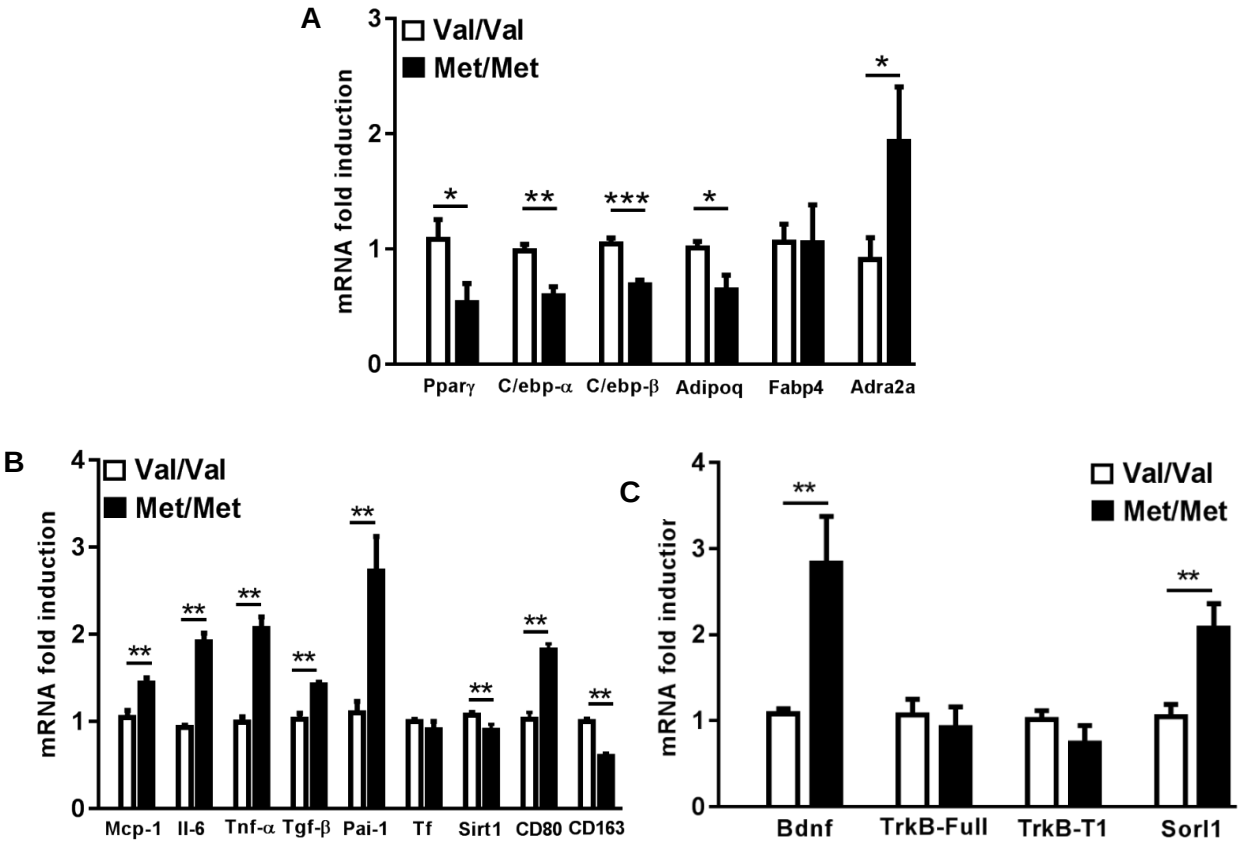


Figure 3. Gene expression profile of epididymal white adipose tissue (epiWAT) in BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. mRNA levels of genes related to (A) adipogenesis, (B) inflammation and (C) BDNF/TrkB pathway in epididymal white adipose tissue (epiWAT) of BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. Data are expressed as mean ± SEM. n = 6 mice/group. Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

2 Evaluation of the role of mutant BDNFVal66Met protein on adipogenesis.

To understand the impact of the mutated BDNF Val66Met protein on adipogenesis, *in vitro* studies were performed. Pre-confluent C3H10Ts1/2 murine mesenchymal stem cells were stimulated with ProBDNFVal or ProBDNFMet synthetic peptides and then the adipogenic program was induced.

Flow cytometry analysis revealed that ProBDNFMet treatment is able to decrease the percentage of cells with low granularity (non-induced; R1) and increased those with high granularity (R4) both at 3 and 9 days post-induction (Figure 4A). In addition, similar levels of lipid accumulation, evaluated with Oil-Red-O staining, were found on day 9 when comparing the cells treated with the two peptides (Figure 4B).

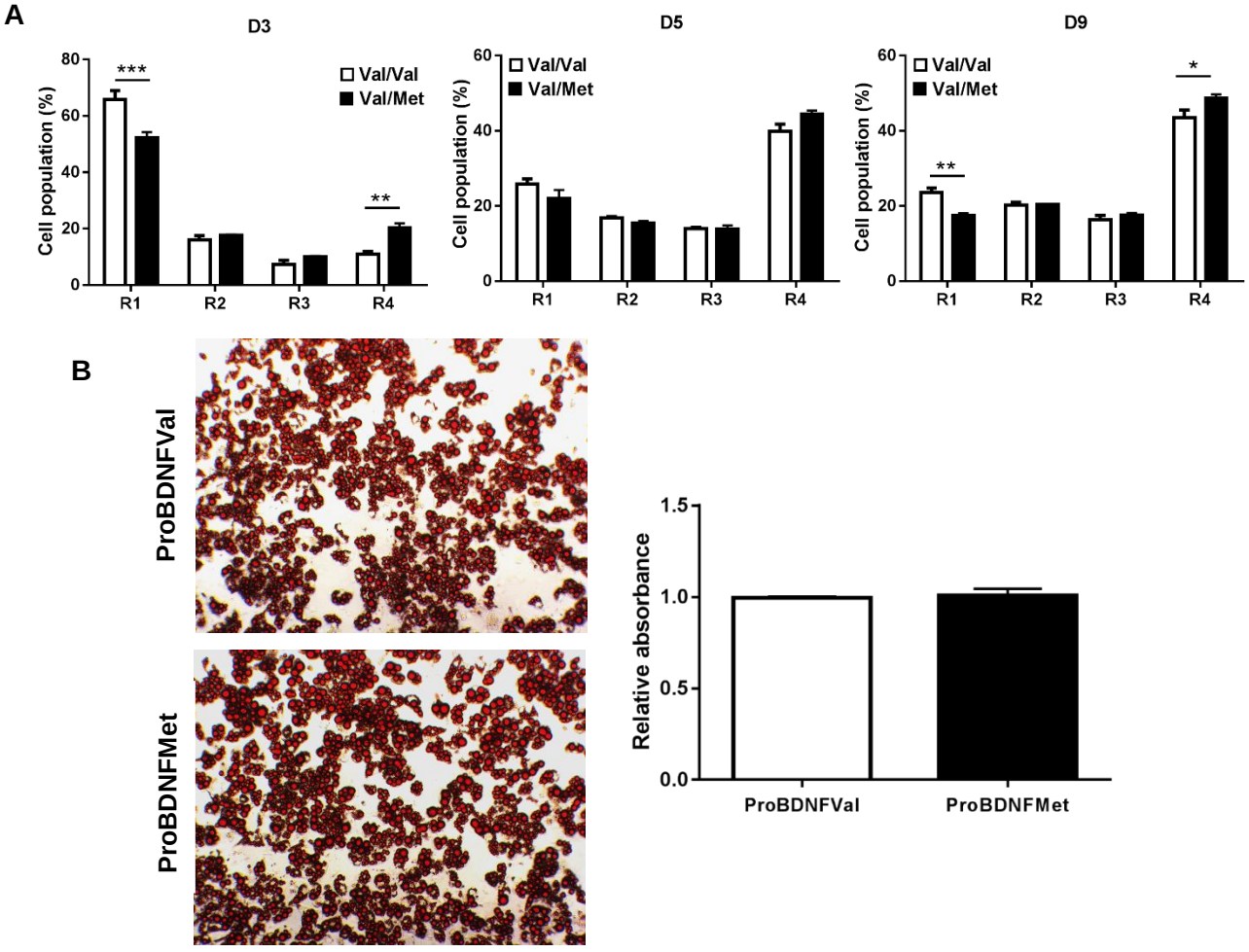


Figure 4. Effect of proBDNFMet on adipogenic differentiation of C3H10T1/2 cells. (A) Percentage of different cells population based on their granularity profile analyzed by flow cytometry (R1: noninduced, R2-R3: growing granularity, R4: high granularity) on day 3 (D3), day 5 (D5) and day 9 (D9) of differentiation. (B) Representative images of Oil-Red-O staining and absorbance measurement in C3H10T1/2 cells. Data are expressed as mean ± SEM. n = 5 independent experiments/group. Student's t-test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$.

Interestingly, the analysis of the expression profile of the genes Pparγ, C/ebp-α, and C/ebp-β, which are involved in adipogenesis, showed a reduction in their level in fully differentiated cells (day 9) after treatment with ProBDNFMet.

In this experimental condition, among the genes that were previously modulated in epiWAT of $BDNF^{Met/Met}$ mice, only Sorl1 was enhanced by the ProBDNFMet treatment at late stages of differentiation (day 9) (Figure 3A and Figure S1).

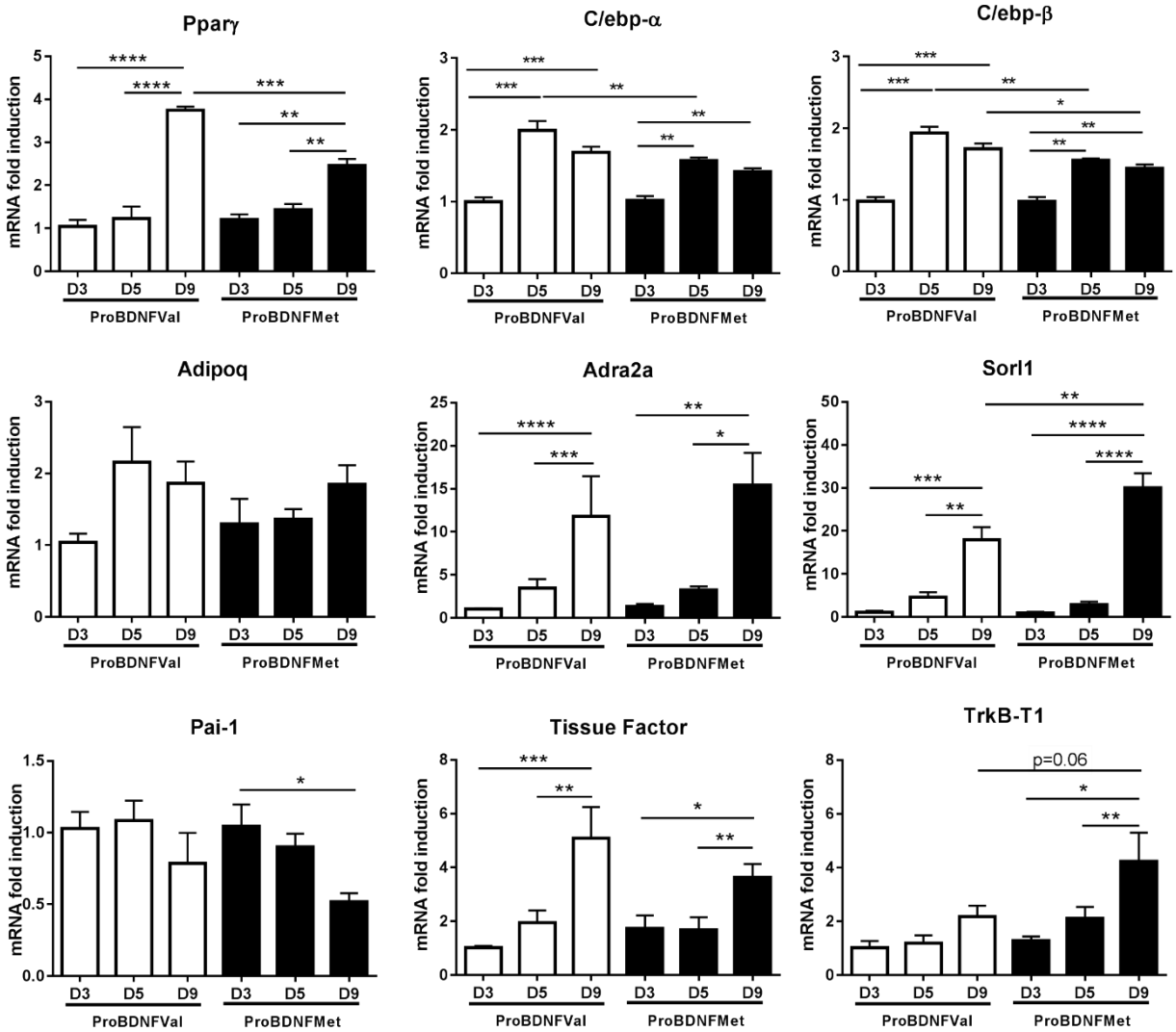


Figure 5. Effect of proBDNFMet on adipogenic differentiation of C3H10T1/2 cells. Analysis of the transcript levels of genes involved in adipogenesis. Data are expressed as mean ± SEM. n = 5 independent experiments/group. Two-way ANOVA followed by Bonferroni post hoc analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

3 Effect of physical exercise on adipose tissue phenotype of BDNFVal66Met mice

According to International cardiovascular guidelines that recommend regular PE as management for the prevention and treatment of CVD, we evaluated the potential beneficial effect of PE on adipose tissue and on the pro-thrombotic phenotype in BDNF Val66Met knock-in mice.

$BDNF^{Val/Val}$ and $BDNF^{Met/Met}$ mice underwent 4 weeks of free voluntary exercise in cages equipped with a running wheel. As previously reported, no difference in the daily running distance was found between $BDNF^{Val/Val}$ and $BDNF^{Met/Met}$ mice ($BDNF^{Val/Val}$: 6.676 ± 0.720 Km and $BDNF^{Met/Met}$ 6.657 ± 0.602 Km; p = 0.9837). In addition, we showed that PE did not affect the percentage of ingWAT and epiWAT on the total body weight in both $BDNF^{Val/Val}$ and $BDNF^{Met/Met}$ mice, compared to sedentary mice (Figure 6).

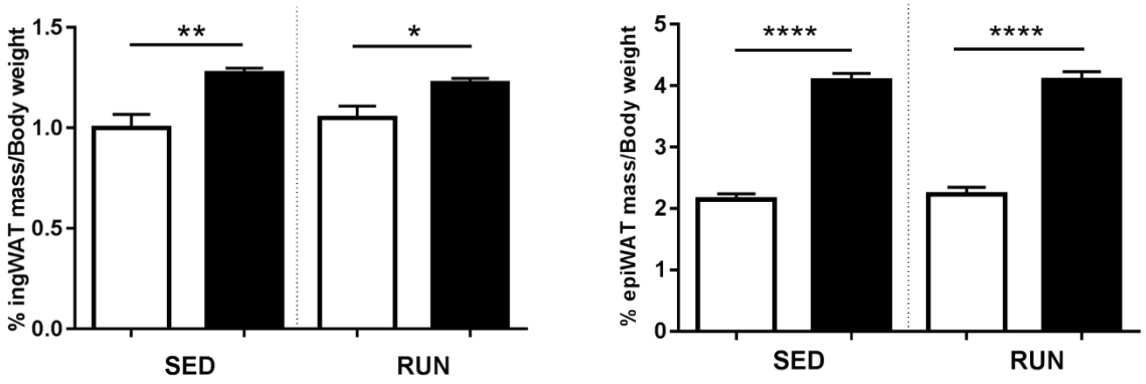


Figure 6. Impact of free voluntary exercise on the mass of adipose tissue depots. (A) Inguinal (ingWAT) and (B) epididymal (epiWAT) white adipose tissue mass on total mouse body weight. Data are expressed as mean ± SEM. n = 6 mice/group. Two-way ANOVA followed by Bonferroni post hoc analysis. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.001$.

PE-induced a change in the profile of the frequency distribution of adipocyte sizes in the ingWAT of both genotype. However, this effect was more evident in $BDNF^{Val/Val}$ than in $BDNF^{Met/Met}$ mice (Figure 7A). Interestingly, in the epiWAT, $BDNF^{Val/Val}$ running mice displayed a significant enrichment in small-size adipocytes and a reduction in medium-size ones compared to sedentary mice, whereas $BDNF^{Met/Met}$ mice showed an opposite trend, even if less marked (Figure 7B).

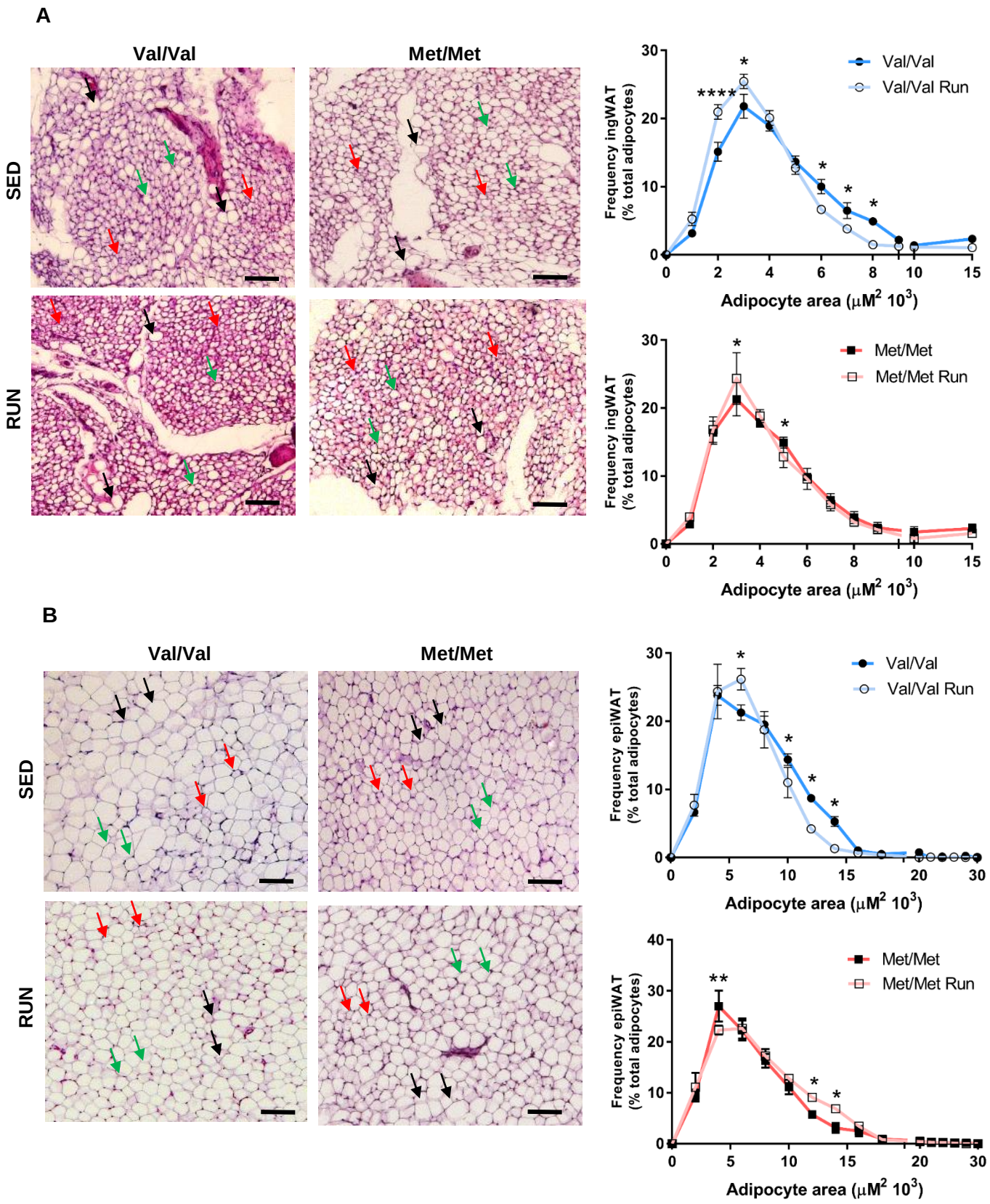
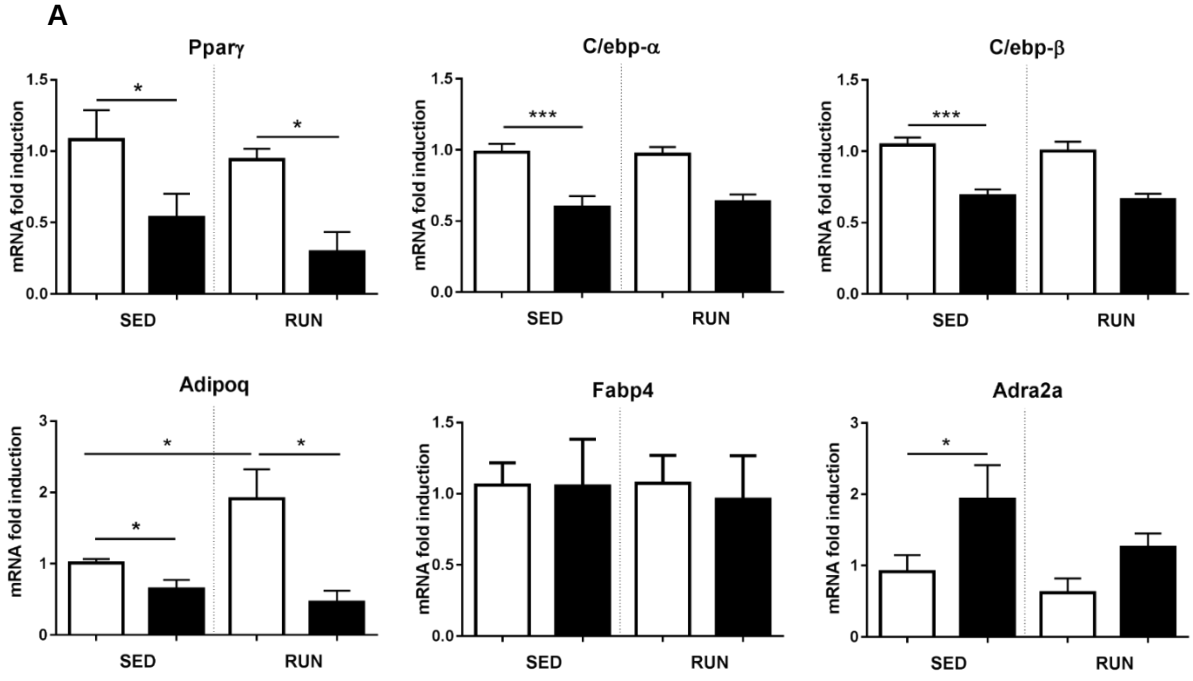


Figure 7. Impact of free voluntary exercise on epiWAT morphology. Representative hematoxylin and eosin (H&E) staining images and analysis of the frequency distribution of adipocyte sizes in (A) ingWAT and (B) epiWAT. Data are expressed as mean ± SEM. n = 6 mice/group. Student's t-test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$.

Notably, PE strongly influenced the gene expression profile of epiWAT. In particular, in BDNF$^{Val/Val}$, 4 weeks of PE enhanced the mRNA level of Adipoq, whereas it did not modify the expression of genes involved in the adipogenic program (Figure 8A) and into inflammation compared to the sedentary ones. In BDNF$^{Met/Met}$ mice, PE was not sufficient to affect the expression of adipogenic genes, but it was sufficient to improve the inflammatory profile, decreasing the expression of Il-6, Tnf-α, Tgf-β, Mcp-1 and Pai-1 (Figure 8B), and to switch the M1/M2 macrophage polarization, reducing the expression of CD80 and increasing the expression of CD163, (Figure 8C).

In addition, the expression of Sorl1 was markedly reduced by PE in both BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice, whereas Adra2a and Sirt1 were only slightly but not significantly decreased in BDNF$^{Met/Met}$ running mice (Figure 8A and 8B). PE modulated conversely the BDNF expression in the two groups of mice. In particular, BDNF mRNA levels increased in BDNF$^{Val/Val}$ running mice and reduced in BDNF$^{Met/Met}$ running when compared to their respective sedentary controls. Of note, the expression of both TrkB-full length and TrkB-T1 isoform was slightly but not significantly increased in both groups of mice after exercise (Figure 8C).



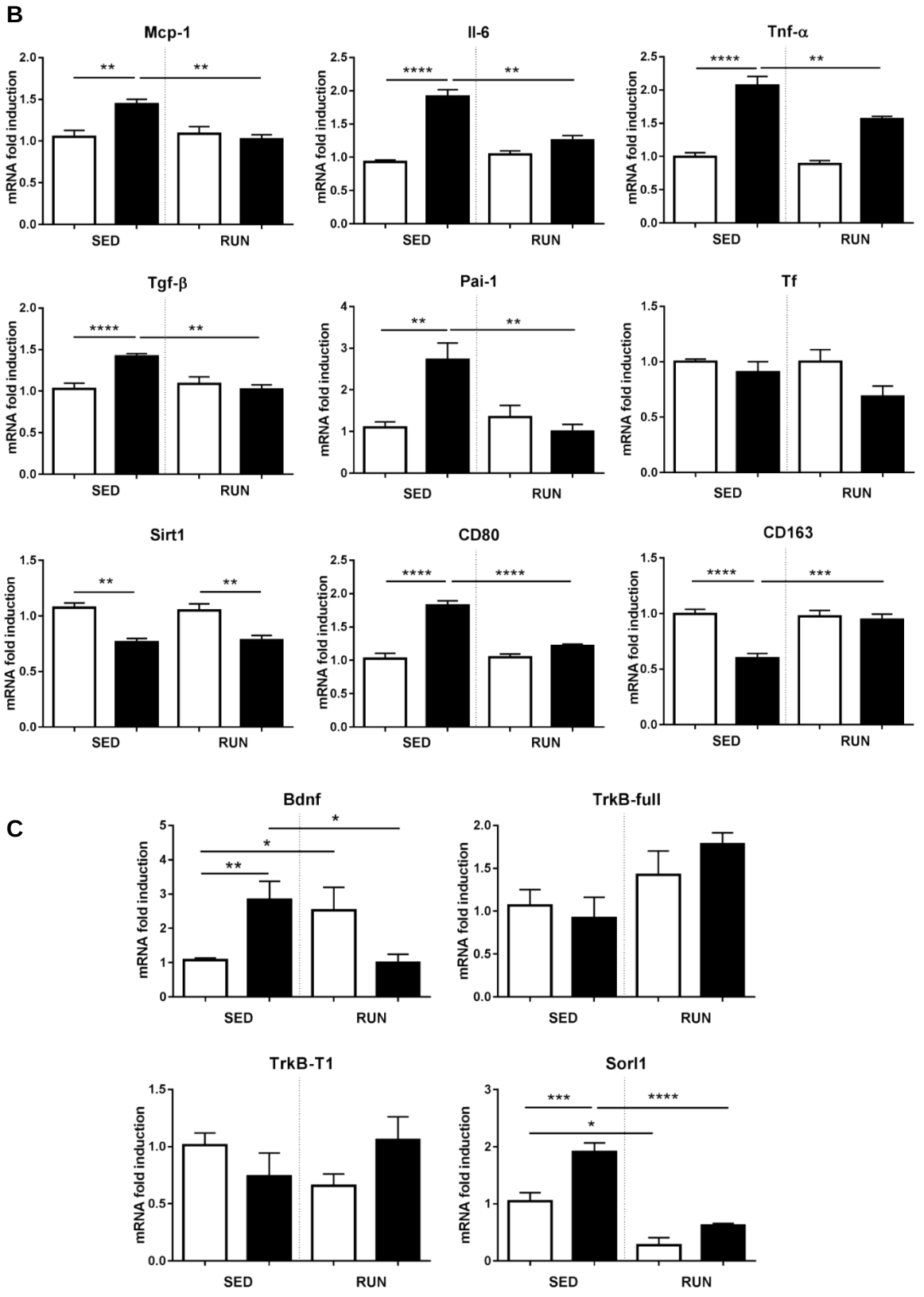


Figure 8. Impact of free voluntary exercise on the gene expression profile of adipose tissue isolated from BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. (A) Adipogenesis, (B) inflammation and (C) BDNF/TrkB pathway-related mRNA levels in epiWAT of sedentary and running BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. Data are expressed as mean ± SEM. n = 6 mice/group. Two-way ANOVA followed by Bonferroni post hoc analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

4 Effect of physical exercise on pro-thrombotic phenotype in BDNF$^{Met/Met}$ mice

Finally, we investigated the ability of 4 weeks of PE to improve the pro-thrombotic phenotype already observed in BDNF$^{Met/Met}$ [23], in terms of platelet and leukocyte aggregates and arterial thrombosis FeCl3-induced.

As previously shown, in the BDNF$^{Met/Met}$ mice there was a higher number of circulating blood cells, platelet activation state and enhanced arterial thrombosis [23]. PE restored the physiological number of platelets and leukocytes, and the natural percentage of platelet/leukocyte aggregates in response to ADP in BDNF$^{Met/Met}$ mice, without affecting significantly these parameters in BDNF$^{Val/Val}$ mice (Figure 9).

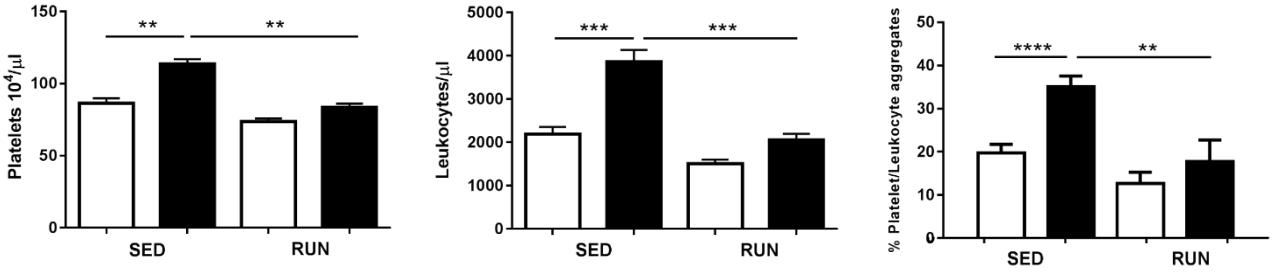


Figure 9. Effect of voluntary physical exercise (PE) on the prothrombotic phenotype of BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. Number of circulating platelets, leukocytes and percentage of platelet/leukocytes in whole blood analyzed by flow cytometry measured in sedentary and running BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. n = 6 mice/group. Two-way ANOVA followed by Bonferroni post hoc analysis. ** $p < 0.01$, *** $p < 0.005$.

$FeCl_3$ application to carotid artery reduced the blood flow in all BDNF$^{Met/Met}$ sedentary mice, leading to a stable occlusion in 100% of mice, whereas only a slight reduction was observed in BNDF$^{Val/Val}$ mice. Of note, PE ameliorated arterial thrombosis, preventing completely the occlusion of the carotid artery in BDNF$^{Met/Met}$ mouse group (Figure 10A and 10B).

Overall, these data show that a paradigm of 4 weeks of free voluntary exercise is able to prevent the pro-thrombotic phenotype of BDNF$^{Met/Met}$ mice.

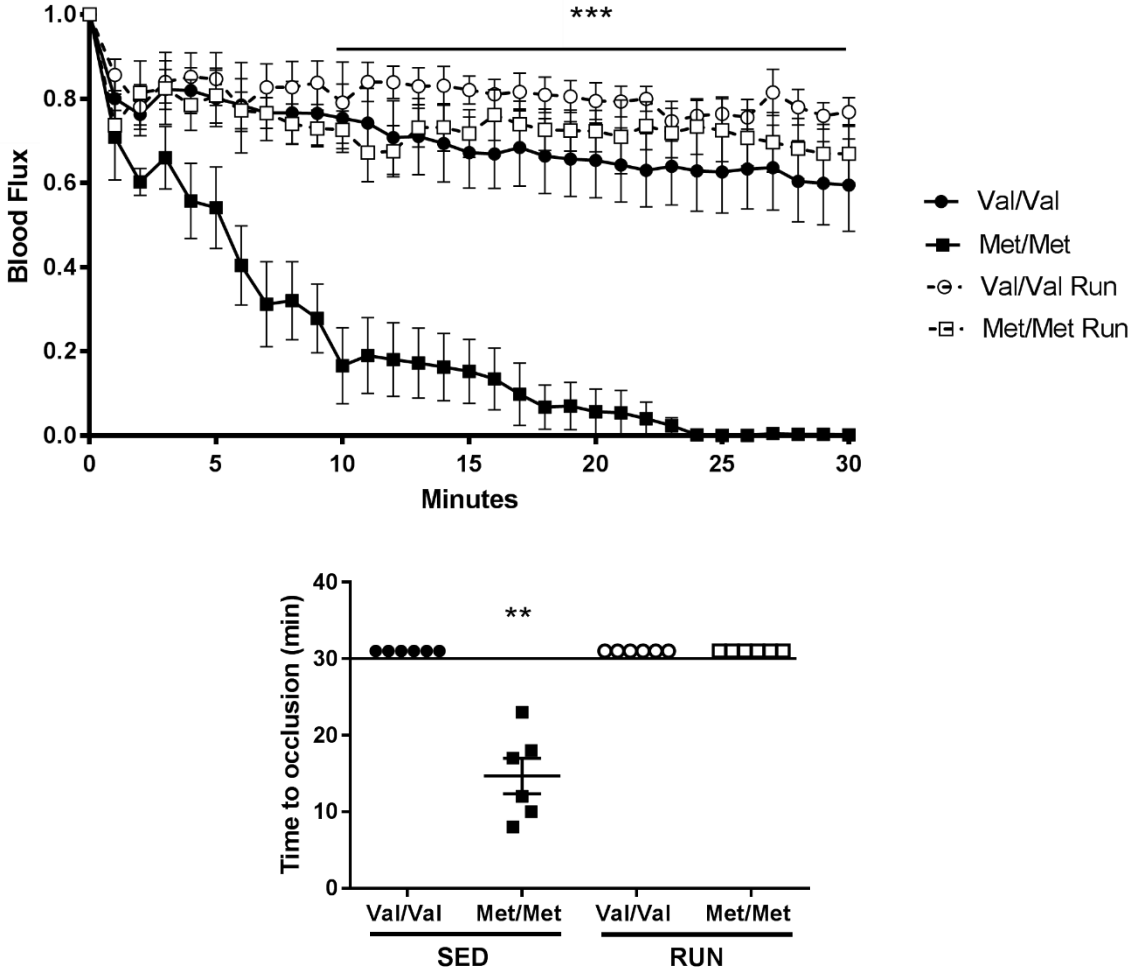


Figure 10. Effect of free voluntary exercise on the pro-thrombotic phenotype of BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. (A) Arterial thrombosis induced by topical application of FeCl3 to the carotid artery and blood flow monitored in sedentary and running BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. (B) Time to thrombotic occlusion. Data are expressed as mean ± SEM. n = 6 mice/group. Two-way ANOVA and Three-way ANOVA with repeated measures followed by Bonferroni post hoc analysis. ** $p < 0.01$, *** $p < 0.00$

4. DISCUSSION I

1 Discussion I

Although mutations, as well as genetic variants of BDNF have been associated with increased in body weight and eating disorders in both human and animal models (Chen et al. 2006, Ieraci et al. 2016, Kernie, Liebl, and Parada 2000, Lebrun et al. 2006, Lyons et al. 1999, Monteleone and Maj 2013, Nakazato et al. 2012, Noble et al. 2011, Rosas-Vargas, Martínez-Ezquerro, and Bienvenu 2011, Speliotes et al. 2010, Thorleifsson et al. 2009), the factors and mechanisms involved still remain to be elucidated. Nowadays, it is known that BDNF-TrkB signaling is an important downstream target of melanocortin 4 receptor (MC4R) pathway involved in the regulation of energy balance and food intake (Rosas-Vargas, Martínez-Ezquerro, and Bienvenu 2011, Xu, Barnes, et al. 2003, Yeo et al. 2004). After confirming literature data showing a higher bodyweight of $BDNF^{Met/Met}$ mice compared to $BDNF^{Val/Val}$, we found that this difference is mainly determined by an increase in the mass of epiWAT and ingWAT. Histological analyses clearly showed a substantial difference in adipocytes size distribution in the epiWAT, with enrichment in small-size cells in $BDNF^{Met/Met}$ mice. The higher epiWAT mass associated with a higher number of small adipocytes, might trace back to hyperplasia, a well-known mechanisms of defense that the adipose tissue can undergo in obesity after a threshold of hypertrophy is reached (Choe et al. 2016, Heilbronn, Smith, and Ravussin 2004, Jernås et al. 2006).

In support of this statement, we observed a higher expression of Adra2a and Sorl1 in the epiWAT of mutant mice. In particular, besides the known anti-lipolytic effect exercised by Adra2a activation, it was demonstrated that Adra2a overexpression is associated with adipose tissue hyperplasia in animal models (Valet et al. 2000). This data was confirmed by human studies were the increased in alpha-/beta-adrenoreceptor ratio, as well as gain-of-function mutations of the Adra2, are associated with obesity (Lafontan et al. 2002, Långberg et al. 2013, Moro et al. 2005). In addition, the alteration in Sorl1 expression suggests the role of this gene in metabolic disease. In particular, the upregulation of the expression of Sorl1, gene coding for the protein Sorla, has been related to reduced lipolytic activity in adipocytes (Schmidt et al. 2016) and GWAS analyses have associated Sorl1 with obesity in humans and in mouse models (Smith et al. 2010, Thorleifsson et al. 2009). The paradigm that overweight and obesity are related to adipose tissue inflammation, that is partially responsible for the systemic low-grade inflammation

found in people affected by these conditions is well established (Ellulu et al. 2017). Of note, we found that the accumulation of epiWAT in BDNF$^{Met/Met}$ mice was accompanied by a higher expression of the M1 pro-inflammatory marker CD80, of the monocyte chemoattractant protein-1 (Mcp-1) and of the mediators of inflammation such as Pai-1, Tnf-alpha, and Il-6.

The connection between adipose tissue inflammation and thrombosis may be well explained by the relationship between the higher levels of inflammatory transcripts and the great number and activation state of circulating leukocytes and platelets.

It is demonstrated that the onset and progression of thrombosis is strictly linked to the presence of inflammatory proteins in the circulation (Berg and Scherer 2005, Bodary 2007, Odrowaz-Sypniewska 2007) that are able to directly enhance platelets activation and leukocytes ability to produce in turn inflammatory factors such as Il-6, Tnf and Cox-2 (Berg and Scherer 2005, Davì et al. 2002, Freedman et al. 2010, Furuncuoğlu et al. 2016, Santilli et al. 2012, Vilahur, Ben-Aicha, and Badimon 2017).

The inflammatory profile observed in BDNF$^{Met/Met}$ mice could also be related to the reduced levels of Pparγ found in BDNF mutant mice. PPARγ, alongside the role of master regulator of adipogenesis, is also involved in the regulation of adipose tissue inflammation being able to down-regulate inflammatory adipokines. Specifically, PPARγ activation down-regulates the expression of inflammatory markers such as MCP-1 and TNFα and thus reduces inflammation in activated macrophages (Li et al. 2004, Okuno et al. 1998, Ricote et al. 1998, Xu, Barnes, et al. 2003). Moreover, PPARγ activation induces adiponectin expression thus further contributing to reducing chronic inflammation (Yamauchi et al. 2001).

Here we showed for the first time that BDNF expression is enhanced in the epiWAT of mutant mice, supporting the hypothesis that BDNF Val66Met polymorphism could contribute to adipose tissue pathophysiology. Previous studies performed using BDNF-(si)RNA-mediated knockdown in the 3T3 cell line showed a reduced adipogenic differentiation ability, supporting the hypothesis that BDNF expression is of functional relevance for the adipogenic commitment. Indeed, after a first phase, BDNF expression is dramatically downregulated during adipocyte differentiation and mature adipocyte was found to contribute only marginally to the neurotrophin production in the adipose tissue (Bernhard et al. 2013).

Interestingly, we showed that the treatment of C3H10T1/2 cells with Pro-BDNFMet before the induction of cell commitment is able to well recapitulate the expression profile of genes found to be altered in the epiWAT of $BDNF^{Met/Met}$ mice.

The reduction of Pparγ and the up-regulation of Sorl1 expression induced by Pro-BDNFMet went along with the increased percentage of mature adipocytes evaluated by flow cytometry, suggesting a clear role of the BDNF polymorphism in adipogenesis. However, Pro-BDNFMet was not able to affect Adipoq and Adra2a as well as Pai-1 expression, let us hypothesize that the entire process of adipogenesis may involve factors deriving from the stromal vascular cells fraction. Indeed, it is suggested that mesenchymal progenitor/stem cells, preadipocytes, endothelial cells, pericytes, T cells, and macrophages and not mature adipocytes are the main source of adipokines and PAI-1 in adipose tissue, and that the stromal vascular fraction in the adipose tissue increases with increasing degree of obesity (Cancello and Clément 2006). Adipose tissue accumulation represents an independent and modifiable risk factor for CVD (Ortega, Lavie, and Blair 2016), and recently the European Guidelines of cardiology strongly recommended regular PE as a valuable management strategy for the prevention and treatment of CVD and metabolic disorders (Lee, Jackson, and Blair 1998, Piepoli et al. 2016).

In the present study, we demonstrated that in mice carrying the BDNF Val66Met human polymorphism, four weeks of PE were sufficient to positively modulate epiWAT morphology and inflammatory profile with an associated reversion of the pro-thrombotic phenotype. Of note, PE was demonstrated to have an important role in the morphological changes observed in the adipose tissue, that can be ascribed to the improvement of the metabolic profile and lipolysis exerted by exercise through the reduction of the expression of Sorl1 and Adra2a (Polak et al. 2005, Schmidt et al. 2016, Stanford and Goodyear 2016).

PE was demonstrated to have a positive effect not only by reducing adipose tissue accumulation (Woods, Vieira, and Keylock 2009, You et al. 2013), especially the visceral one (Bruun et al. 2006, Vieira et al. 2009), but also reducing chronic inflammation associated with different diseases (Goh, Goh, and Abbasi 2016).

The beneficial effect of PE was found to be mediated by its ability to decrease mitochondrial dysfunction and to reduce oxidative stress through AMPK and PGC-1α pathway (Kjøbsted et al. 2018, Lira et al. 2010), with a direct reduction of adipokines released from the adipose tissue. This reduction goes alongside with a

decrease of macrophage infiltration through the reduction of Toll-like receptors on monocytes and macrophages, that is able to limit macrophage M1 polarization (Goh, Goh, and Abbasi 2016). PE action is also mediated by the induced release of anti-inflammatory molecules from skeletal muscle and leukocytes (Leal, Lopes, and Batista 2018).

We showed that PE in BDNF$^{Met/Met}$ mice is able to decrease the transcript levels of inflammation mediators, to reduce classical activation (M1) of macrophages, and negatively affect the number of circulating leukocytes and platelets. These modifications are able to ameliorate the pro-thrombotic phenotype observed in mutant mice. Interestingly, the expression of Bdnf was found to be differently modulated by PE in the two genotypes, with an increase in its levels in BDNF$^{Val/Val}$ mice and a decrease in mutant mice. These results may be related to the different adipose tissue morphology found in the two genotypes, suggesting an important correlation between adipocyte dimension and BDNF levels. In fact, a higher percentage of small adipocytes was associated with high levels of Bdnf (e.g. sedentary BDNF$^{Met/Met}$ and running BDNF$^{Val/Val}$), and vice versa, since low levels of the transcript were measured when the mean adipocyte dimension was higher (e.g. sedentary BDNF$^{Val/Val}$ and running BDNF$^{Met/Met}$).

The difference observed might be due to the different impact of the stromal vascular cell fraction involved during adipocytes turnover and also to the different contribution of the peripheral nervous system (Kim et al. 2014, Rigamonti et al. 2011, White and Ravussin 2019). In line with this hypothesis, it must be underlined that PE failed to enhance the mRNA of Bdnf in the central nervous system of BDNF$^{Met/Met}$ mice (Ieraci et al. 2016) with possible consequences also on the levels of BDNF transcript in the peripheral nervous system. Along with this, it is worth to be mentioned that, in opposite to data presented here regarding the beneficial effect of PE on adipose tissue inflammation and thrombosis, the same BDNF$^{Met/Met}$ murine model did not show beneficial neurobiological changes induced by exercise (Ieraci et al. 2016).

5. RESULTS II

1 Sub-Chronic Restraint Stress (RS) induced activation of the hypothalamic-pituitary-adrenal axis and altered body weight

To evaluate the possible impact of the BDNFVal66Met polymorphism on the response of the hypothalamic-pituitary–adrenal (HPA) axis during chronic stress, $BDNF^{Val/Val}$ and $BDNF^{Val/Met}$ mice underwent 7 days of sub-chronic restraint stress (RS). Both $BDNF^{Val/Val}$ and $BDNF^{Val/Met}$ stressed mice displayed a higher adrenal glands/body weight ratio, parameter that was used as a proxy of HPA axis activation and stress response. When compared to controls no differences were found between the genotypes, suggesting an equivalent response to stress (Figure 1A). Of note, 7 days of RS induced a progressive reduction in the bodyweight of $BDNF^{Val/Val}$ mice, while in $BDNF^{Val/Met}$ mice, after a reduction during the first 4 days of stress, the weight returned to basal levels during the following days (Figure 1B)

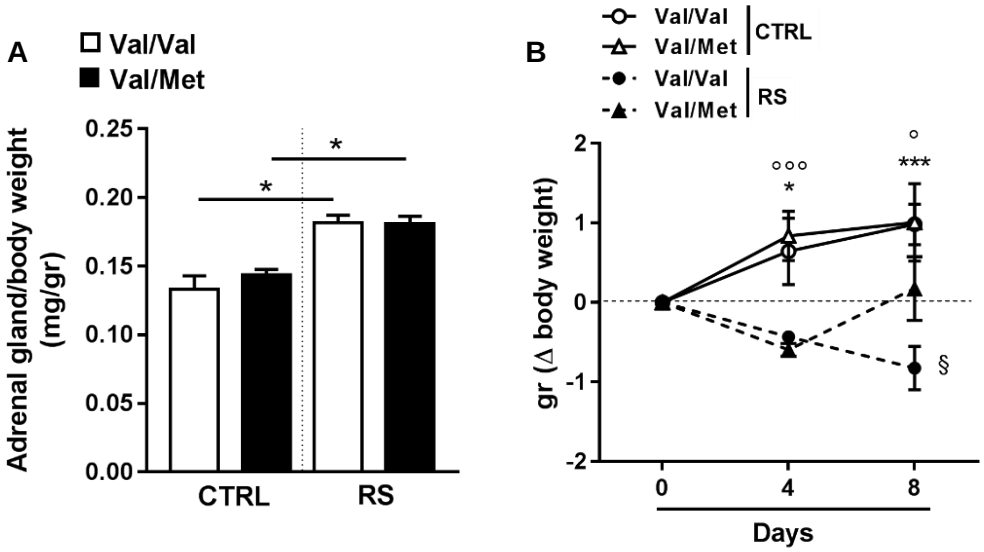


Figure 1. Evaluation of stress response in $BDNF^{Val/Val}$ and $BDNF^{Val/Met}$ mice. Mice underwent Sub-chronic stress (RS) treatment for 7 days, 2 h/day. (A) Adrenal gland weight to bodyweight ratio. (B) Delta (Δ) body weight of stressed (RS) and not stressed (CTRL) $BDNF^{Val/Val}$ and $BDNF^{Val/Met}$ mice at day 4 and day 8. Data are expressed as mean ± SEM. n = 8 mice/group. Two-way ANOVA and three-way ANOVA with repeated measures followed by Bonferroni post hoc analysis, p values were obtained by using log-transformed variables. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.005$ $BDNF^{Val/Val}$ (CTR) vs. $BDNF^{Val/Val}$ stressed (RS) mice, °°° $p < 0.005$ $BDNF^{Val/Met}$ (CTR) vs. $BDNF^{Val/Met}$ stressed (RS) mice and §§§§ $p < 0.00015$ $BDNF^{Val/Val}$ stressed (RS) vs. $BDNF^{Val/Met}$ stressed (RS) mice.

2 RS predisposed BDNF$^{Val/Met}$ mice to thrombosis

It was previously shown that chronic stress is able to increased depressive-like and anxiety-like behaviours and to impair working memory in BDNF$^{Val/Met}$ mice through the hyperactivation of the HPA axis. Since the continuous stimulation of the HPA axis is known to be a potential mechanism by which stress may increase the risk of arterial thrombosis, we wanted to evaluate the possible interaction between stress and BDNF polymorphism regarding the thrombotic profile. To do this, we evaluated arterial thrombus formation *in vivo* after the topical application of $FeCl_3$ to the exposed carotid artery. BDNF$^{Val/Met}$ stressed mice displayed a significant reduction in the blood flow soon after the starting of the measurements, while only a slight effect was observed in BDNF$^{Val/Val}$ stressed mice (Figure 2A). In line with this, a total occlusion (flow reduction >90%) was reached only in stressed BDNF$^{Val/Met}$ mice after an average time of 20 min (Figure 2B).

Overall, these data show that sub-chronic stress is able to elicit the pro-thrombotic phenotype of BDNF$^{Val/Met}$ mice, while heterozygous Met allele *per se* is not sufficient to predispose to this condition.

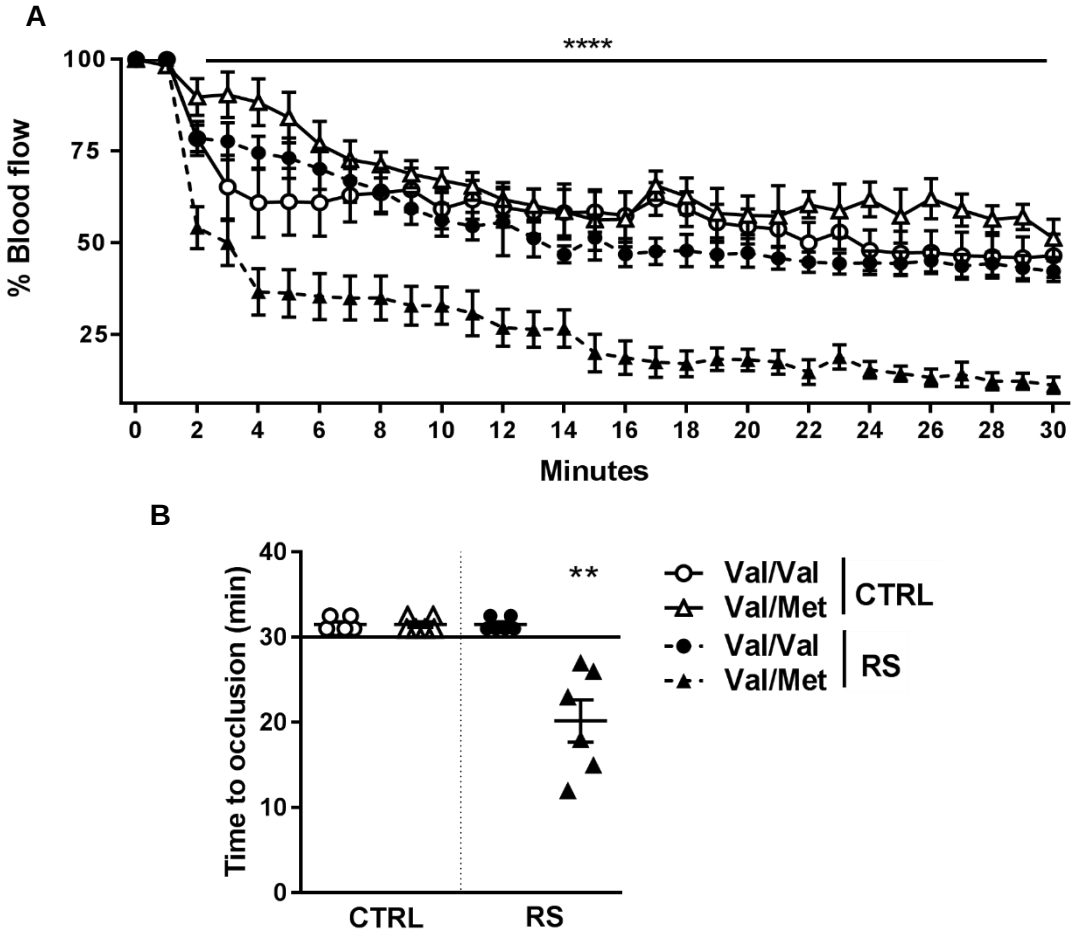


Figure 2. Effect of restraint stress on arterial thrombosis in BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice. Arterial thrombosis was induced by the topical application of FeCl3 to the carotid artery in mice. (A) Blood flow in the carotid arteries of stressed (RS) and not stressed (CTRL) BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice groups. (B) Time to thrombotic occlusion. Data are expressed as mean ± SEM. n = 6 mice/group. Two-way ANOVA and Three-way ANOVA with repeated measures followed by Bonferroni post hoc analysis, p values were obtained by using log-transformed variables. **p < 0.01 and **** p < 0.001.

3 RS increased circulating blood cell number and platelet activation state

The analyses of circulating blood cells and platelet function in our experimental protocol showed the main genotype effect between BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice regarding those parameters.

RS increased the number of leukocytes (Figure 3A) and platelets (Figure 3B), as well as the percentage of reticulated platelets (Figure 3C) in both BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice when compared to the control littermates (CTR). Remarkably, the effect of RS was more pronounced in BDNF$^{Val/Met}$ platelets than in BDNF$^{Val/Val}$ (Figure 3B, C).

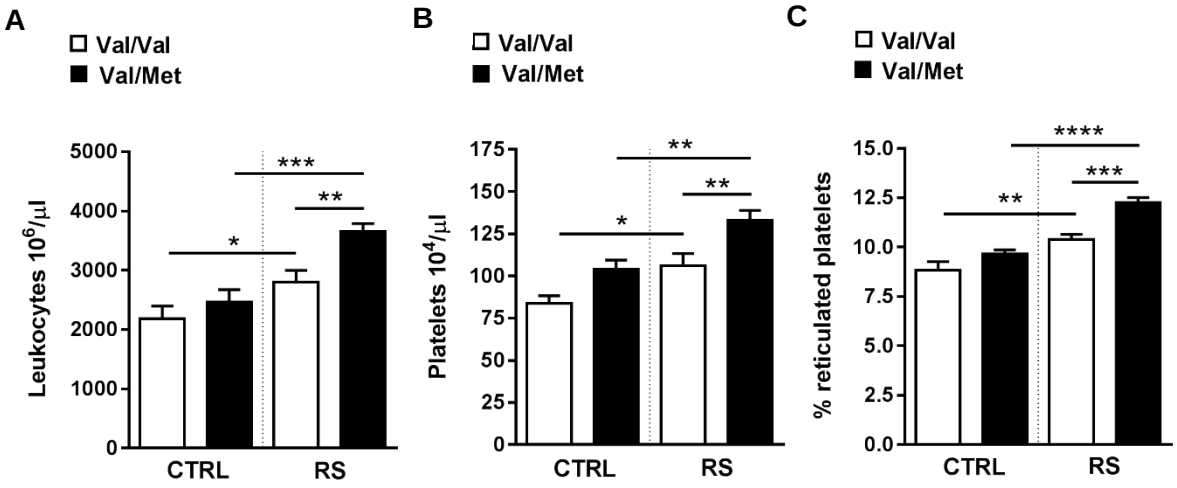


Figure 3. Effect of restraint stress on circulating blood cell number. Numbers of circulating (A) leukocytes and (B) platelets. (C) Percentage of reticulated platelets analysed by flow cytometry in stressed (RS) and not stressed (CTRL) BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice. Data are expressed as mean ± SEM. n = 6 mice/group. Two-way ANOVA followed by Bonferroni post hoc analysis, p values were obtained by using log-transformed variables. * p < 0.05, ** p < 0.01, *** p < 0.01and **** p < 0.001.

In addition, while in platelets the activation of integrin αIIbβ3 (GPIIbIIIa) (Figure 4A) and the expression of P-selectin (Figure 4B) in response to thrombin (THR) and ADP of BDNF$^{Val/Val}$ stressed mice was comparable to mice not exposed to RS, platelets from BDNF$^{Val/Met}$ stressed mice displayed a significant hyper-reactivity.

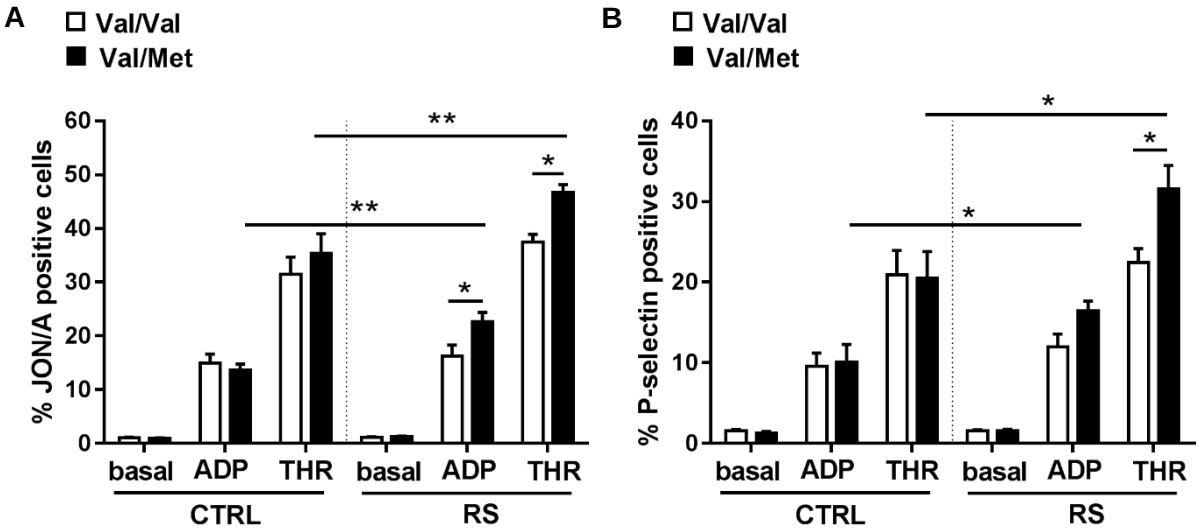


Figure 4. Effect of restraint stress on platelet activation in BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice. (A) GPIIbIIIa activation (JON/A-PE antibody) and (B) P-selectin expression was evaluated by flow cytometry analyses in washed platelets at basal condition or after exposure to ADP (5 µM) or thrombin (THR 0.05 U/mL) from stressed (RS) and not stressed (CTRL) BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice. Data are expressed as mean ± SEM. n = 6 mice/group. Two-way ANOVA followed by Bonferroni post hoc analysis, p values were obtained by using log-transformed variables. * $p < 0.05$ and ** $p < 0.01$.

In addition, the percentage of platelet/leukocyte aggregates in response to ADP was enhanced in both BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice after RS when compared to their control littermates. However, this parameter was more markedly increased in stressed BDNF$^{Val/Met}$ mice compared to both BDNF$^{Val/Met}$ mice after RS and BDNF$^{Val/Met}$ control mice (Figure 5).

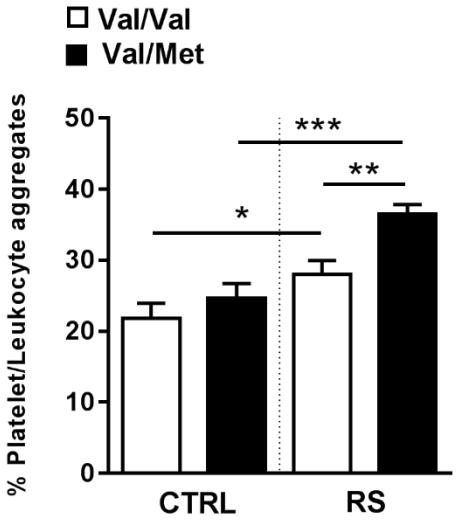


Figure 5. Effect of restraint stress on platelet/leukocyte aggregates in BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice. Percentage of platelet/leukocytes in whole blood isolated from stressed (RS) and not stressed (CTRL) BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice. Data are expressed as mean ± SEM. n = 6 mice/group. Two-way ANOVA followed by Bonferroni post hoc analysis, p values were obtained by using log-transformed variables. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

4 RS increased bone-marrow megakaryocytes number without affecting their maturation state

To understand if the change in the number of platelets observed after RS derived from an alteration of the megakaryopoiesis, we performed immunocytochemistry analysis on the femur bone marrow.

Analysis of the sections of femur bone marrow showed that the number of megakaryocytes (MKs) is enhanced in both stressed BDNF$^{Val/Val}$ and BDNF$^{Val/Met}$ mice (Figure 6 A and B). Nonetheless, MKs had similar dimensions (Figure 6C) and nuclear complexity (Figure 6D) and the analysis of size distribution showed a similar distribution in the mononucleated, binucleated, or polynucleated cell subpopulations among all the analyzed groups.

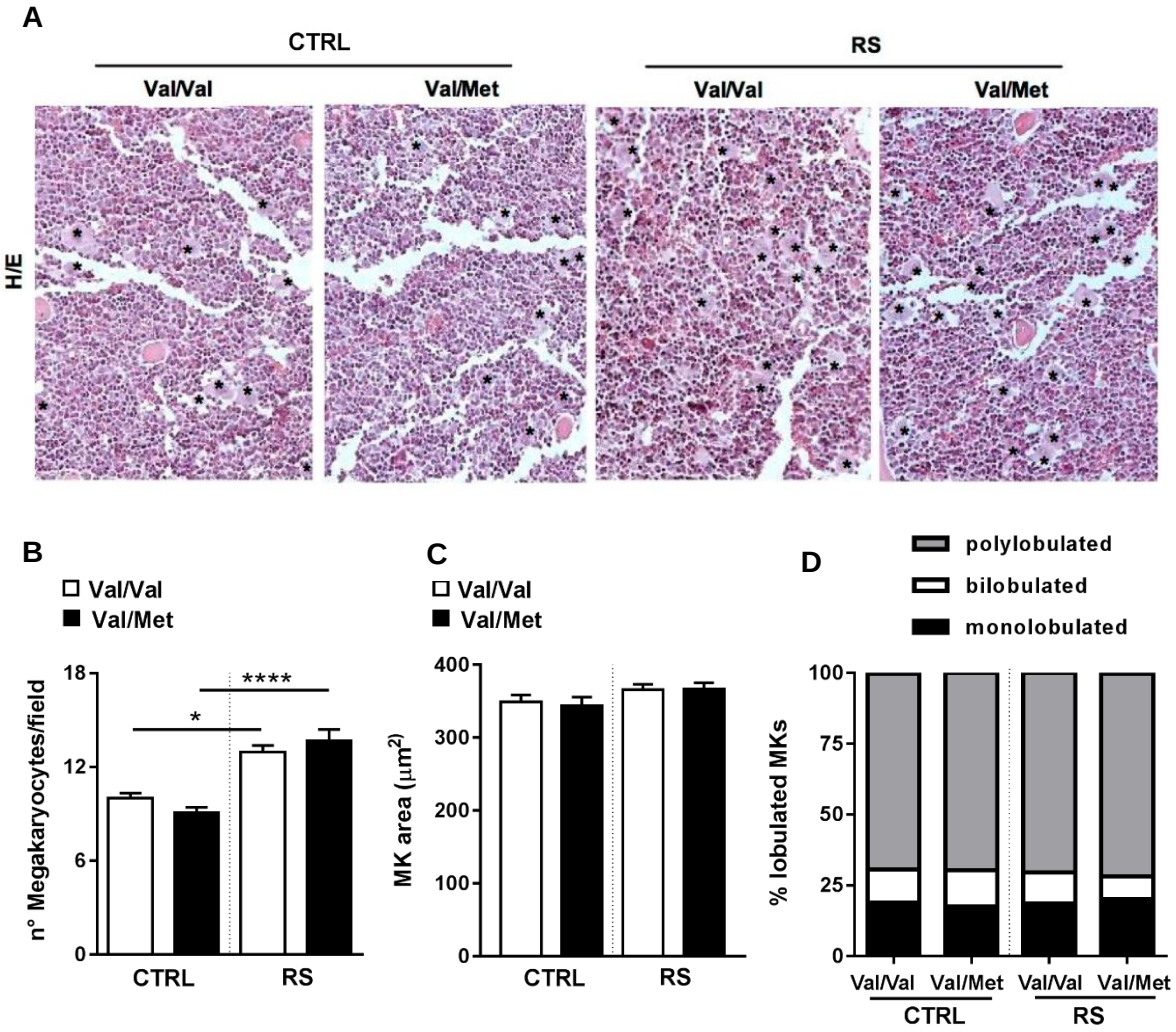


Figure 6. Effect of restraint stress on bone-marrow megakaryocytes. (A) Hematoxylin and Eosin (H&E) staining of bone marrow. Asterisks indicate MKs; (B) quantification of panel (A) expressed as megakaryocytes per field (40× magnification). Analysis of (C) area and of (D) nuclear complexity in megakaryocytes.from stressed (RS) and not stressed (CTRL) $BDNF^{Val/Val}$ and $BDNF^{Val/Met}$ mice. Data are expressed as mean ± SEM. n = 8 mice/group. Two-way ANOVA followed by Bonferroni post hoc analysis, p values were obtained by using log-transformed variables. * $p < 0.05$ and **** $p < 0.001$.

5 RS altered the expression of Tissue Factor and BDNF but not the expression of Sirt1

Since tissue factor (TF) activity is known to be modulated by stress (Stämpfli et al. 2014), we decided to examine its expression and activity in carotid artery tissue. Interestingly, while TF activity was enhanced in both $BDNF^{Val/Val}$ and $BDNF^{Val/Met}$ stressed mice in carotid tissue (Figure 7A), RS induced TF mRNA expression only in $BDNF^{Val/Met}$ mice (Figure 7B). The analysis of Sirt1 mRNA expression, known to be an important modulator of TF and arterial thrombosis [29,30] did not find significant changes among all the groups analyzed (Figure 7C).

Interestingly, we found that BDNF expression was comparable between $BDNF^{Val/Met}$ and $BDNF^{Val/Val}$ mice, and RS reduced its expression in both genotypes but this decrease was greater in $BDNF^{Val/Val}$ mice (Figure 7D).

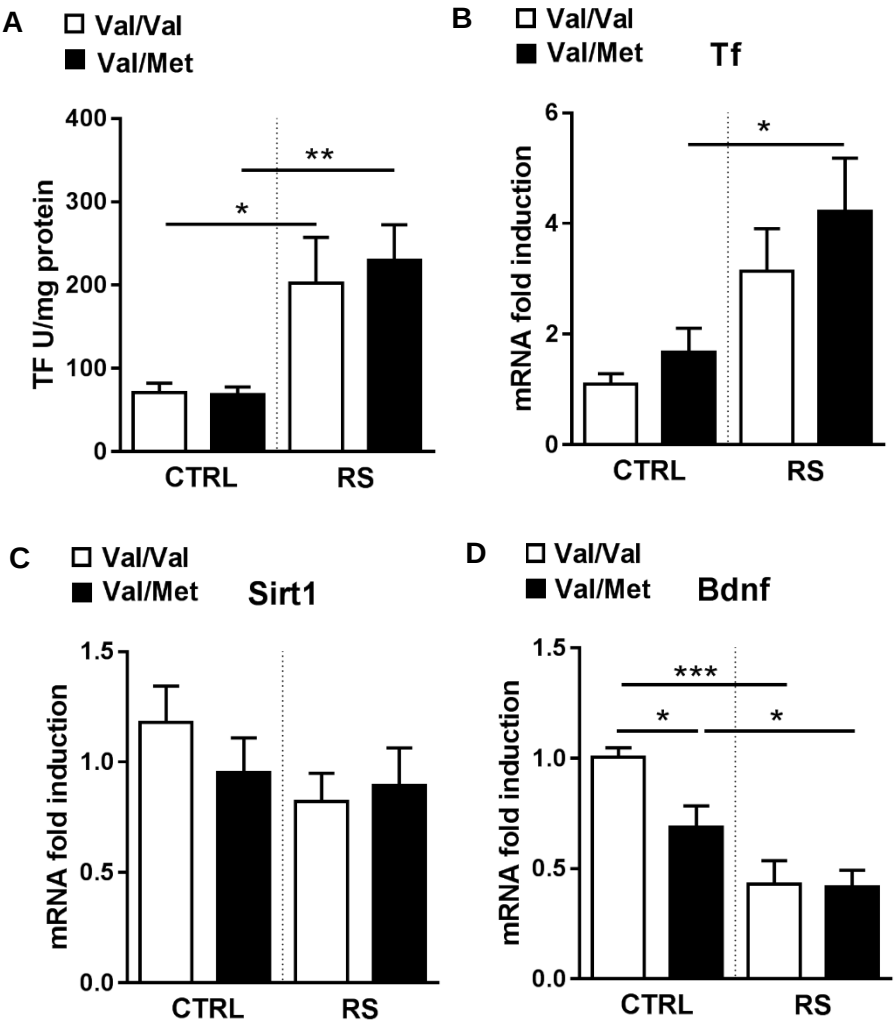


Figure 7. Impact of restraint stress on Tissue Factor, BDNF and Sirt1 expression in arterial tissue. (A) Tissue Factor (TF) activity and (B) TF, (C) Sirt1, (D) BDNF mRNA levels in arterial tissue of stressed (RS) and not stressed (CTRL) $BDNF^{Val/Val}$ and $BDNF^{Val/Met}$ mice. Data are expressed as mean ± SEM. n = 8 mice/group. Two-way ANOVA followed by Bonferroni post hoc analysis, p values were obtained by using log-transformed variables. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$.

6. DISCUSSION II

1 Discussion II

The interaction between genes and environmental (GxE) factors were widely studied in the last few years, discovering that many multifactorial diseases, including CVD, may result from these relations (Svensson et al. 2017, Bondy 2007, Elosua 2018).

Of note, genetic variants of some neurotrophins, including the BDNF Val66Met, were found to make the carriers more susceptible to stressors (Hashimoto, Shimizu, and Iyo 2004). In line with this, a recent meta-analysis of gene-environment interaction showed an interaction between life stress and BDNFVal66Met polymorphism in relation to depression (Zhao et al. 2018). Here, we provide evidence that seven days of RS, already reported to promote anxiety/depressive-like phenotype in heterozygous BDNF Val66Met ($BDNF^{Val/Met}$) mice (Yu et al. 2012), are sufficient to unveil arterial thrombosis in $BDNF^{Val/Met}$ mice through the enhanced platelet activation and coagulation pathway.

It has been hypothesized that the higher propensity to anxiety and depressive related disorder (Yu et al. 2012) observed in Met carriers may be due to dysfunctions in adrenergic and HPA axis (Alexander et al. 2010) and recently it was demonstrated that Met polymorphism has an influence on the cortisol responsivity to stress, thereby implying a Met-allele role in BDNF and cortisol integrative system (de Assis and Gasanov 2019). Indeed, a significant reduction in hippocampal volume (Frodl et al. 2007) and the hyperactivation of the amygdala (Montag et al. 2008) are observed in both Met allele carriers and in subjects under stressful conditions (Geuze, Vermetten, and Bremner 2005, Ressler 2010). In particular, hippocampal neurons are known to exert an inhibitory effect on the activation of the HPA axis, whereas the activity of the amygdala exerts a significant excitatory effect on the axis let hypothesizing common mechanism at the bases of these pathologies. A recent meta-analysis demonstrated that BDNF and cortisol systems are integrated by the glucocorticoid receptors dynamics and that BDNF and cortisol undoubtedly play distinct and complementary roles in the physiology of the nervous system in which cortisol proves to be the regulator of positive as well as negative effects (de Assis and Gasanov 2019). It is well known that the activation of HPA axis under stressful condition promotes dysregulation of both norepinephrine (McEwen 2007) and coagulation system (e.g., tissue factor and the plasminogen activation inhibitor-1) (Uchida et al. 2012, Yamamoto et al. 2002), and affects platelet number (Chen et

al. 2016, Sandrini et al. 2017) and functionality (Stämpfli et al. 2014, Dong et al. 2015, Heidt et al. 2014), thus enhancing the risk of arterial thrombosis.

Interestingly, the increased activity of the amygdala has been recently associated with increased risk of CVD events and with increased activity of the bone marrow (Tawakol et al. 2017).

Here we observed that, according to previous data obtained from both human and animal models, sub-chronic stress is sufficient to enhance the number of bone marrow MKs, circulating leukocytes and platelets and to induce platelet activation (Chen et al. 2016, Sandrini et al. 2017, Heidt et al. 2014).

All these alterations are strongly and significantly increased in stressed $BDNF^{Val/Met}$ mice and, surprisingly, well recapitulate the phenotype observed in non-stressed $BDNF^{Met/Met}$ homozygous mice (Amadio, Colombo, et al. 2017).

Recently, Stämpfli et al. showed that acute RS is able to induce arterial thrombosis in mice without affecting the activity and the expression of TF in the vessel wall (Stämpfli et al. 2014). Of note, we observed that in $BDNF^{Val/Val}$ mice sub-chronic RS increases TF activity without modifying mRNA, suggesting that protracted stress may able to modulate TF in a post-transcriptional manner. Remarkably, both TF expression and activity were positively modulated in $BDNF^{Val/Met}$ mice under sub-chronic stress. However, if it was demonstrated that in $BDNF^{Met/Met}$ mice TF expression was positively modulated following a reduction of Sirt1 activity (Amadio, Colombo, et al. 2017), no effect was observed on this gene after sub-chronic stress in all experimental groups considered. In line with this evidence, experiments will be performed to understand which pathways are involved in the modulation of TF induced by protracted stress in order to unveil new possible targets.

It is well known that both BDNF Met allele variant and stressful conditions are associated with lower expression of BDNF in the central nervous system (Yu et al. 2012, Chao 2003). However, no data are available about the impact of this polymorphism and/or stress in other tissues. We show that BDNF mRNA levels are only slightly but not significantly reduced in the arterial tissue of $BDNF^{Val/Met}$ mice compared to $BDNF^{Val/Val}$.

In addition, sub-chronic RS induced a reduction in BDNF expression in both $BDNF^{Val/Met}$ and $BDNF^{Val/Val}$, even if it was significant only in $BDNF^{Val/Val}$. This suggests that a certain basal level of expression in arterial tissue is necessary, supporting previous data showing the fundamental role of this neurotrophin in the

development and maintenance of the vasculature (Caporali and Emanueli 2009, Donovan et al. 2000, Kermani and Hempstead 2007).

Finally, in line with a previous study of chronic RS (Jeong, Lee, and Kang 2013), our data showed a weight reduction in stressed $BDNF^{Val/Val}$ mice along the experimental time. As previously found, the weight loss observed during the stress can be explained by an early decrease in food intake and subsequently to an increased in energy expenditure and in body temperature (Jeong, Lee, and Kang 2013). In contrast, in $BDNF^{Val/Met}$ mice we observed a weight loss only in the first few days of RS, followed by a weight gain, with a return to basal levels on the eighth day.

This trend might be explained by the critical role of BDNF in the regulation of food intake and body weight control (Lebrun et al. 2006) since BDNF is an anorexigenic factor (Lebrun et al. 2006). Low levels of BDNF in the hippocampus and dorsal-vagal complex (Rios 2011), as well as mutation in the BDNF gene (Lyons et al. 1999, Coppola and Tessarollo 2004, Gray et al. 2006), are associated with hyperphagia, weight gain, and obesity. On these premises, we hypothesize that sub-chronic stress may unmask the hyperphagic phenotype in $BDNF^{Val/Met}$ mice reversing the initial weight loss by enhancing food intake.

7. CONCLUSIONS

1 Conclusions

Despite the advances in prevention and treatment strategies, cardiovascular diseases (CVDs) still remain the leading cause of morbidity and mortality worldwide. It was recently reported that conventional risk factors for CVDs are predictors of the majority, but not the totality, of morbidity and mortality, raising the awareness on the need to identify new dynamics involved in the development of onset and progression of these pathologies (Stamler, Wentworth, and Neaton 1986). Interestingly, environmental factors such as stress, depression, and anxiety were recently included as new risk factors for CVDs for their ability to modulate not only the onset and progression of CVDs but also the response to therapies (Cohen, Edmondson, and Kronish 2015). The emerging role of the impact of lifestyle behavior on CVDs lead to coin the term "behavioral cardiology" as "an emerging field of clinical practice based on the recognition that adverse lifestyle behaviors, emotional factors, and chronic life stress can all promote atherosclerosis and adverse cardiac events" (Rozanski et al. 2005) . In particular, the role of the new discipline would be a deeper understanding of the pathophysiology of behavior-related CVDs and the development of effective therapeutic interventions both to modify high-risk lifestyles and behaviors and to reduce psychosocial risk factors for patients. In the last years, a growing body of the literature showed that psychosocial pathologies benefit from non-pharmacological interventions such as physical exercise. In particular, it has been shown that regular activity is associated with a better level of self-esteem and mood state and lower stress and anxiety levels. In addition, the 2016 European Guidelines on CVD prevention in clinical practice, strongly recommended regular physical exercise (PE) as management for the prevention and treatment of CVD both in healthy people and patients with metabolic disorders (Piepoli et al. 2016).

Brain-derived neurotrophic factor (BDNF), a protein member of the neurotrophin family, has been considered a suitable candidate to study the cellular and molecular mechanism at the bases of CVDs related to psychosocial disorders. BDNF is highly expressed in the brain and it can be detected also in macrophages, endothelial cells, megakaryocytes, and platelets. Besides its neuropoietic action, BDNF modulates cell survival and differentiation, vascular development and heart function (Donovan et al. 2000). Intriguingly, expression and release of BDNF might be altered by genetic and/or epigenetic modifications which, in turn, may affect BDNF availability and function. In this context, the human BDNFVal66Met variant is known to determine a

reduction of the activity-dependent secretion and signaling of mature BDNF, to associate with neuropsychiatric disorders, anxiety and a higher susceptibility to stress (Tsai 2018), and to contribute to the individual propensity for arterial thrombosis related to AMI (Amadio, Colombo, et al. 2017) and to eating disorders and obesity in humans (Beckers et al. 2008, Zhao et al. 2014). Interestingly, a knock-in mouse carrying the human BDNFVal66Met polymorphism represents a good model of all these pathologies observed in human patients showing a depression-like/anxiety-related behavior and a significantly higher body weight than wild-type littermates (Chen et al. 2006), associated with a pro-inflammatory and pro-thrombotic phenotype (Amadio, Colombo, et al. 2017).

Interestingly, stressful conditions unveil the anxious/depressive-like behavioral phenotype in heterozygous BDNFVal66Met ($BDNF^{Val/Met}$) mice, suggesting an important involvement of Met allele in terms of gene-environment interaction (GxE) regarding the behavioral profile. However, the interplay between Met allele and stress in relation to CVD, and in particular to arterial thrombosis, is completely unknown. With this study, we demonstrated that sub-chronic stress is sufficient to unveil also the prothrombotic phenotype in BDNFVal/Met mice affecting the number and functionality of blood circulating cells, and the expression of key thrombotic molecules in arterial tissue. Moreover, it is known that the homozygous Met mice have an impairment in the beneficial neurobiological changes observed with exercise. However, no data are available regarding the effect of physical exercise on the thrombotic risk (Sandrini et al. 2018).

In vitro data showed in this study support the role of Pro-BDNFMet in adipogenesis in line with data obtained in the white adipose tissue of $BDNF^{Met/Met}$ mice. Spontaneous physical exercise is able to induce positive morphological changes and reduce the inflammatory profile of the adipose tissue. These beneficial effects might be at the bases of the observed reduction in the pro-thrombotic phenotype detected in this animal model.

This study supports the important interaction between both positive and negative environmental factors and the Met allele of the BDNF gene in relationship to arterial thrombosis showing a new possible gene-environment interaction (GxE). Human studies will be crucial to confirm this possible gene-environment interaction and to assess if it needs to be taken into account to deploy better strategies of clinical management of the arterial thrombosis risk in patients carrying this polymorphism.

8. MATERIALS AND METHODS

1 Animal models and in vivo procedures

BDNF Val66Met mice were kindly provided by Francis S. Lee from the Weill Cornell Medicine University (NY). They were generated introducing the human Val66Met mutation in an ApaIApaI fragment containing the BDNF prodomain and put into a targeting vector. To verify protein expression a carboxyl-terminal His tag was inserted using the PCR mutagenesis strategy. The loxP-Neo cassette was introduced into an EagI site as a positive marker of selection. A pGK-thymidine kinase cassette was used as a negative selectable marker. The targeting vector was comprised of a 1.5 kb short arm, a 4.8 kb long arm, a 1.5 kb targeted sequence carrying the Val66Met mutation, and the Neo cassette that was flanked by two loxP sites. Linearized targeting vectors were inserted into 129 mouse strain embryonic stem (ES) cells by electroporation. DNA derived by G418/FIAU-resistant ES clones were screened using a diagnostic BglII + BamHI restriction enzyme digestion using the 5' probes external to the targeting vector sequence. Four positive ES clones were injected into C57BL/6 blastocysts, which were then introduced into pseudo-pregnant females. Chimeric animals were mated with C57BL/6 to produce heterozygous animals, and these mice were subsequently crossed with mice expressing Cre-Recombinase in germ cells to excise the neo cassette. BDNF $^{Val/Val}$ mice, heterozygous ($BDNF^{Val/Met}$) and homozygous ($BDNF^{Met/Met}$) mice for Met/Met mutation were generated by interbreeding heterozygotes.

All animals were housed in a temperature-controlled, 12 h light/dark cycle environment with ad libitum access to water and fed on a standard pellet diet. All experiments were approved by the National Ministry of Health-University of Milan Committee and of DGSA (N° 12/2012, 12/2015 and 349/2015). All surgical procedures were performed in mice anesthetized with ketamine chlorhydrate (75 mg/kg; Intervet) and medetomidine (1 mg/kg; Virbac).

1.1 Genotyping

Offsprings were genotyped by PCR analysis of ear punch-derived genomic DNA using the following primers:

Val R (Val) 5’-CTCTTCGATGACGTGCTCAA-3’

Primer S (Val/Met) 5’-TCATACTTCGGTTGCATGAAGG-3’

His R (Met) 5’-ATAAATCCACTAGTGGTGGTGG-3’

1.2 Restraint Stress procedure

Sub-chronic stress was induced by restraint stress (RS) test performed as previously described (Yu et al. 2012). Briefly, $BDNF^{Val/Val}$ and $BDNF^{Val/Met}$ mice were divided randomly into stressed (RS) and control (CTRL) groups. RS was performed daily for 2 h for 7 consecutive days, in well-ventilated polypropylene restrainers. At the end of the stress session, mice were returned to their home cage. CTRL mice were handled for 2 minutes and then returned to the home cage. All mice had free access to food and water during the study. Mice were weighed and sacrificed 24 h after the last session of restraint stress.

1.3 Free Voluntary exercise protocol

Mice underwent a free voluntary exercise protocol as already described (Ieraci et al. 2016). Briefly, $BDNF^{Val/Val}$ and $BDNF^{Met/Met}$ mice were allocated randomly into running and control groups in cages equipped with or without running wheels, respectively, for 4 weeks with free access to food and water.

2 Arterial Thrombosis Model

For the experimental arterial thrombosis model, the left carotid artery of anesthetized mice was dissected free and placed in the probe (model 0.7V, Transonic System) connected to a transonic flow meter (Transonic T106). After blood flow was stabilized (baseline flow constant for 7 min at least 0.8 ml/sec), a 1 × 1 mm strip of filter paper (Whatman N°1) soaked with $FeCl_3$ (10% solution; Sigma-Aldrich) was applied over the carotid artery. After 3 min, the filter paper was removed, the carotid artery was washed with PBS, and the flow was recorded for 30 min. An occlusion was considered to be total and stable when the flow was reduced by >90% from baseline until the 30-minute observation time, with the flow during this period not changing by more than 1% from baseline per second.

3 Blood Analyses

3.1 Whole Blood Counts

Blood was collected into 3.8% sodium citrate (1:10 vol: vol) from anesthetized mice by cardiac venipuncture, and the differential white blood cell and platelet count were performed on a Beckman Coulter AU480.

3.2 Platelet–Leukocyte Aggregate Analysis

Platelet/monocyte and platelet/neutrophil aggregates were analysed as previously described (Amadio, Tarantino, et al. 2017). Briefly, citrated blood was stimulated where indicated with ADP for 5 minutes and red blood cells were lysed by FACS Lysing solution (BD Biosciences); samples were stained with the anti-CD45, anti-CD41, and anti-CD14 or anti-Lys6G (eBiolegend, Cat. 103101, 133901, 150101, and 127601, resp.) and analysed by flow FACS "Novocyte 3000." A minimum of 5000 events was collected in the CD14+ or Lys6G+ gate.

3.3 Platelet Studies

Washed platelets (WPs) were obtained from platelet-rich plasma (PRP), isolated following centrifugation at 100 for 10 min of citrated blood as previously described (Barbieri et al. 2015), with serial centrifugation and addition of 0.2 mM PGI2 and 0.01 mg/l apyrase. Platelet pellets were resuspended in HEPES-Tyrode's buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 0.35% bovine serum, 1 mM $MgCl_2$, 2.7 mM KCl, and 3.3 mM NaH_2PO_4).

25 µl of WPs (5×10^4/µl in HEPES-Tyrode's buffer supplemented with 1 mM $CaCl_2$) was mixed with a saturating concentration of PE-conjugated JON/A (Emfret Analytic, Cat. M023-2) antibody, raised against the activated form of GPIIbIIIa (αIIβIII integrin), or with anti-CD62P and FITC-conjugated antibody (P-selectin; BD Biosciences, Cat. 553744), and the mixture reacted with different concentrations of ADP or thrombin for 15 minutes at room temperature. The reaction was stopped by 400 µl ice-cold PBS, and samples were analysed within 30 minutes. Platelets were identified by forward and side scatter distribution and by anti-CD41 positivity.

Reticulated platelets (RP) were identified by the thiazole orange method (Barbieri et al. 2015): 10 µl of PRP was incubated with 390 µl of thiazole orange (Retic-Count; BD Biosciences) or PBS as control and anti-CD41 at room temperature for 10 minutes, in the dark. Immediately after incubation, samples were analysed by flow cytometry collecting 10000 CD41-positive events; the percentage of RP was recorded, and the absolute number of RP was calculated by multiplying by the platelet count.

4 Bone marrow Analyses

4.1 Megakaryocyte histology

Immunohistochemistry was performed on BM and adipose tissue. Tissues were fixed overnight in 4% formalin, embedded in paraffin, cut at 3 µm, and mounted on polarized slides. Before paraffin embedding, BM samples were decalcified in 10% EDTA, pH 8 for 10 days. BM and adipose tissue sections were stained in Hematoxylin and Eosin (H&E). The number and area of megakaryocytes were evaluated in hematoxylin and eosin-stained sections by counting 5–7 40x microscopic fields for each tissue sample (Trakala et al. 2015). The number and size of adipocytes were evaluated in hematoxylin and eosin-stained sections by counting five 5× microscopic fields for each tissue sample using the ImageJ-Macro Adipocytes Tool.

All images were digitalized by a Zeiss Axioskop (Carl Zeiss) equipped with an intensified charge-coupled device (CCD) camera system (Photometrics).

5 Measurement of TF Activity in Aortic Tissue

Aorta samples were lysed with 15 mM n-Octyl-B-d-glucopyranoside lysis buffer at 37 °C for 10 min, sonicated at 20 kHz for 20 s and diluted with 25 mM HEPES saline. The total protein concentrations of the homogenates were determined using the Bradford method. 40 µL of carotid artery homogenate (1.25 µg/uL), or vehicle were mixed with 40 µL citrated pooled wild-type mouse plasma and 40 µL $CaCl_2$ (final concentration 5 mM), and procoagulant activity was quantified by a one-stage plasma recalcification time assay (Chlopicki et al. 2004). Clotting times were expressed in relative units/µg protein based on a standard curve of serially diluted human thromboplastin preparation. Plasma deficient of Factor VIIa as well as preincubation with specific TF-neutralizing antibody was used to demonstrate TF dependence of procoagulant activity.

6 Cell culture, treatment, and differentiation

C3H10T1/2 were cultured in DMEM medium supplemented with 100 U/mL penicillin (Gibco, Rodano, Milan, Italy), 100 µg/mL streptomycin (Gibco, Rodano, Milan, Italy) and 10% FBS, at 37°C in 5% CO_2/95% air atmosphere. Cells were plated in 6 well plates at a concentration of $3.5x10^4$ cells/ml, and when reached the 80% of confluence (day -2) were treated with 10 ng/ml of ProBDNFVal or ProBDNFMet synthetic peptide (Alomone Labs, Jerusalem, Israel). Forty-eight hours later (day 0), cells were treated

with adipogenic commitment mix (5 µg/ml Insulin, 2 µg/ml Dexamethasone, 0.5 mM IBMX and 5 µM Rosiglitazone; all from Cayman Chemical, Arcore, Italy). Insulin (5 µg/ml) was added at day 3, 5 and 7 until day 9 when the complete differentiation of the cells was reached.

7 Adipogenesis evaluation by flow cytometry and Oil-Red-O

After ProBDNFVal or ProBDNFMet treatment, C3H10T1/2 cells were analyzed during adipogenesis by flow cytometry as previously described (Lee et al. 2004). Briefly, at day 3, 5 and 9 cells were harvested in ice-cold PBS and analyzed by flow cytometry and according to granularity (SSC-H) divided into four categories that correlate with the increased level of cell lipids accumulation after adipogenic commitment. In particular, non-induced cells were detected into the R1 gate, while cells with increasing granularity were identified in the regions from R2 to R4.

Oil-Red-O staining was performed as already described (Kraus et al. 2016) on day 9. Lipid content was quantified as absorbance at a wavelength of 518 nm using a Tecan Infinite M1000 plate reader spectrophotometer (TECAN, Männedorf, Switzerland).

8 Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from adipose tissue or C3H10T1/2 cells with TRIzol Reagent (Sigma-Aldrich, Saint Louis, MO, USA) and Direct-zol RNA extraction kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. One µg of RNA was reverse-transcribed using iScript Advanced cDNA Synthesis Kit (Bio-Rad Laboratories, Segrate, Milan, Italy). qPCR was then carried out using the primer sequences shown in the table.

Table 1. Primers sequences of the analysed genes.

Gene	Sequence			Gene	Sequence	
Gapdh	fwd	CGTGCCGCCTGGAGAAACC		Tgf-β	fwd	TACTATGCTAAAGAGGTCACCC
	rev	TGGAAGAGTGGGAGTTGCTGTTG			rev	CTTCCCGAATGTCTGACGTATTG
Pparγ	fwd	GCCCTTTGGTGACTTTATGGA		Pai-1	fwd	GTCTTTCCGACCAAGAGCAG
	rev	GCAGCAGGTTGTCTTGGATG			rev	ATCACTTGGCCCATGAAGAG
C/ebp-α	fwd	AGAGCCGAGATAAAGCCAAAC		Tf	fwd	CCCAAACCCGTCAATCAAGTC
	rev	TCATTGTCACTGGTCAACTCC			rev	CCAAGTACGTCTGCTTCACAT
C/ebp-β	fwd	CCCCGCGTTCATGCA		CD163	fwd	GGGTCATTCAGAGGCACACTG
	rev	CAGTCGGGCTCGTAGT			rev	CTGGCTGTCCTGTCAAGGCT
Adipoq	fwd	AGGCATCCCAGGACATC		CD80	fwd	CTGGGAAAAACCCCCAGAAG
	rev	CCTGTCATTCCAACATCTCC			rev	TGACAACGATGACGACGACTG
Fabp4	fwd	GGCGTGGAATTCGATGAA		Sorl1	fwd	GTGTGAGGACGGCGAGGCAT
	rev	GCTTGTCACCATCTCGTT			rev	GGTGGACTGCTGCCTCTGGTCA
Adra2a	fwd	AAAACCTCTTCCTGGTGTCC		Sirt1	fwd	AGCAGGTTGCAGGAATCCAA
	rev	CGCTTCAGGTTGTACTCGAT			rev	CACGAACAGCTTCACAATCAACTT
Il-6	fwd	AAGACAAAGCCAGAGTCCTTCAGAG		Bdnf	fwd	TCGTTCCTTTCGAGTTAGCC
	rev	GTCTTGGTCCTTAGCCACTCCTTC			rev	TTGGTAAACGGCACAAAAC
Mcp-1	fwd	CTTCTGGGCCTGCTGTTCA		Trk Full	fwd	CTCAAGTTGGCGAGACATTCCAAG
	rev	CCAGCCTACTCATTGGGATCA			rev	GGGGGTTTTCAATAACAGGAATCT
Tnf-a	fwd	TGCCTATGTCTCAGCCTCTTC		TrkB-T1	fwd	TGGTGATGTTGCTCCTGCTC
	rev	GAGGCCATTTGGGAACTTCT			rev	CCCATCCAGTGGGATCTTATGA

Samples of cDNA were incubated in 15 µL Luna® Universal qPCR mix containing the specific primers and fluorescent dye SYBR Green (New England Biolabs, Pero, Milan, Italy). RT-qPCR was carried out in triplicate for each sample on the CFX Connect real-time System (Bio-Rad Laboratories, Segrate, Milan, Italy) as previously described (Sandrini et al. 2018). Gene expression was analyzed using parameters available in CFX Manager Software 3.1 (Bio-Rad Laboratories, Segrate, Milan, Italy).

9 Adipose Tissue Histology and Quantification of Adipocyte Size and Number

Immunocytochemistry and analysis of adipocytes were performed in inguinal (ingWAT) and epididymal (epiWAT) white adipose tissue. Tissues were fixed overnight in 4% formalin, embedded in paraffin, cut at 5µm, and mounted on polarized slides. The number and size of adipocytes were evaluated in hematoxylin and eosin-stained sections by counting five 5x microscopic fields for each tissue sample using the ImageJ-Macro Adipocytes Tool. Images were taken with a Zeiss Axioskop (Carl Zeiss, Milan, Italy) equipped with an intensified charge-coupled device (CCD) camera system (Photometrics, Tucson, AZ, USA).

10 Statistical Analyses

Statistical analyses were performed with GraphPad Prism 6.0 and SAS versus 9.4 software (SASA Institute). Data were analyzed by Student's t-test, one, two or three-way ANOVA with repeated measures for main effects of treatment and time or stimuli when necessary, followed by a Bonferroni post hoc analysis as appropriate. Values of <0.05 were considered statistically significant. Data are expressed as mean ± SEM.

9. BIBLIOGRAPHY

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