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PhD THESIS

EMERGING ROLE OF NEUROTROPHINS IN CARDIOVASCULAR REGULATION:

IMPACT AND CHARACTERIZATION OF GENETIC VARIANT BDNF (Val66Met) POLYMORPHISM ON THROMBOTIC EVENTS

BIO/14

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1 INTRODUCTION

1 Main causes of cardiovascular disease and thrombosis

Cardiovascular disease (CVD) is a broad term for a range of diseases affecting the heart and blood vessels (capillaries and veins) (Anthea, 1993).

Specifically it refers to any disease that affects the cardiovascular system, principally cardiac disease, vascular diseases of the brain and kidney, and peripheral arterial disease (Kelly, 2010).

CVD is the leading causes of deaths worldwide, through since the 1970s, cardiovascular mortality rates have declined in many high-income countries (Institute of Medicine of the National Countries, 2010). At the same time, cardiovascular deaths and disease have increased at a fast rate in low- and middle-income countries (Mendis, et al., 2011). It is expected that by 2020, CVD would prevail as the leading cause of death and disability over infectious diseases globally (Murray, et al., 1997). Although very important progress in the treatment and prevention of these diseases has been made, intense contemporary research efforts are aiming to unravel new diagnostic tools (Campos, et al., 2013).

The cardiovascular system is regulated by the autonomic nervous system, the renin-angiotensin-aldosterone system, nitric oxide (NO) and other factors including neuropeptides (Wong, et al., 2012).

Over past three decades, the field of medicine has made a drastic progress in diagnosis, prevention and treatment of CVD. The Framingham Heart Study and the Seven Countries' Study were the two major studies that made significant contribution in identifying major risk factors for CVD (Murray, et al., 1997) (Farouhi, et al., 2006). Substantial body of evidence, supporting the reduction of conventional risk factors, led to the search of new emerging risk factors. Over 300 conventional risk factors for CVDs have been discovered which mostly fall into two broad categories: ***a***) modifiable and ***b***) non-modifiable. Modifiable risk factors (i.e. high blood pressure, high blood cholesterol, tobacco use, diabetes mellitus and obesity) (Kelly, 2010) are the factors that if treated and controlled would reduce the CVD risk, while non-modifiable risk factors (i.e. genetic factors) could not be modified to reduce the CVD burden (Smith, et al., 2000) (Expert panel on detection, 2001). Predictive models, based on conventional risk factors, were reported as

underutilized and possessed lower accuracy than desired (Brotman, et al., 2005), from this observation derived the necessity to explore new risk factors for CVD.

Emerging risk factors are broadly categorized as either early markers risk or markers of existing disease (Assman, et al., 2005) (Helfand, et al., 2009).

Among early markers there are genetic polymorphisms identified in genes encoding HDL, LDL, triglycerides CRP and body mass index which lead to an increased risk of MI (Thanassoulis DG, 2010).

On the other hand there are C-reactive protein levels, carotid intima-media thickness, lipoprotein (a) levels, apolipoprotein B levels, homocysteine levels, oxidative mediators (such as myeloperoxidase and F2-isoprostane) and vitamin D levels among markers of existing disease (Gupta, et al., 2013).

Recently, several large, prospective, longitudinal studies have revealed that the relation between depression and CVD is significant and independent of conventional risk factors (Jiang, et al., 2002). Indeed, a large body of evidence has emerged to suggest that depressive disorder is a risk factor for heart diseases, both etiologically and prognostically. Actually, depression is associated with changes in an individual's health status that may influence the development and course of cardiovascular disease, including noncompliance with medical recommendations, as well as the presence of cardiovascular risk factors such as smoking and hypertension. In addition, depression is associated with physiologic and pathologic changes, including nervous system activations, cardiac rhytm disturbances, multidistrectual inflammation and hypercoagulability that negatively influence the cardiovascular system. Furthermore, stress may be an underlying trigger that leads to the development of both depression and cardiovascular disease (Joynt, et al., 2003).

Beyond the fact that the risk factors that lead to CVDs are different, CVDs result the major clinical consequence of thrombotic events, in particular of arterial thrombosis, venous thromboembolism (VTE), and disseminated intravascular coagulation (DIC).

Arterial thrombosis is the most common cause of death in the developed world. 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 myocardial infarction (MI) or stroke. Arterial thrombi are platelet-rich and are referred

to as white clots. Various risk factors can increase the incidence of arterial thrombosis, such as smoking, hypertension, hyperlipidemia and diabetes mellitus (Owens, et al., 2010).

Venous thromboembolism (VTE), which is a collective term for both deep-vein thrombosis (DVT) and pulmonary embolism (PE), is the third leading cause of cardiovascular death in the developed world. Commonly formed in the large veins of the leg, these clots are primarily composed of red cells and fibrin. As opposed to arterial thrombosis, which occurs due to arterial injury and exposure of the sub-endothelium, venous thrombosis mainly occurs due to changes in the composition of the blood, changes in blood flow and/or activation of the endothelium (Mackman, 2008) (Eppihimer, et al., 2000). Many different factors can increase the incidence of VTE, such as the presence of cancer, obesity and major surgery (Cushman, 2007) (Urbach, et al., 2003). Thrombophilia is the propensity to develop thrombosis as a result of changes in the blood itself.

Disseminated intravascular coagulation (DIC) is characterized by microvascular thrombosis (Levi, et al., 1999). Sepsis and endotoxaemia are the most common pathologic conditions leading to DIC (Levi, et al., 1999) (Baglin, 1996). DIC can also be caused by severe trauma, such as surgery, resulting in the release of "tissue debris" into the circulation and subsequent activation of coagulation (Levi, 2005) (Levi, et al., 1997) (Gando, 2001).

Although several factors involved in these processes have been identified and targeted successfully with therapeutic interventions (Smith, et al., 2006) (Krumholz, et al., 2006), there are clearly many that remain to be elucidated. Because of the multifactorial nature of thrombosis and the difficulty in elucidating factors involved in human disease, there is a need for *in vivo* animal models to explore the pathophysiolgy of thrombosis.

1.1 Key regulators of thrombosis - Thrombus formation within a vessel is the precipitating event in multiple vascular disease processes, including MI, thrombotic cerebrovascular events and venous thrombosis; however, the pathophysiological processes regulating these diseases are distinct. In venous thrombosis, primary hypercoagulable states, reflecting defects of the proteins of coagulation and fibrinolysis, or secondary hypercoagulable states, involving abnormalities of blood vessels and blood flow, lead to thrombosis (Andreotti, et al., 2005). In contrast, arterial thrombosis is highly dependent on the vessel wall and platelets (Freedman, 2005).

Under normal circumstances, the hemostatic process is a delicate balance of prothrombotic and antithrombotic factors in the vasculature. Normal hemostasis prevents 1) uncontrolled hemorrhage, 2) keeps the blood in a fluid state and 3) removes blood clots after restoration of vascular integrity; however these same pathways regulating hemostasis lead to pathological thrombosis and vessel wall occlusion.

Upon injury, complex processes are activated and involve interaction between the vascular wall, platelets, leukocytes, coagulation proteins, and other as yet unidentified components (Randal, et al., 2007). Specifically the first component that contributes to hemostasis is ***the coagulation system*** with the expression of ***tissue factor***, while the second important component starts with ***platelet activation***, which not only contributes to the hemostatic plug, but also accelerates the coagulation system (Versteeg, et al., 2013) (*Figure 1*).

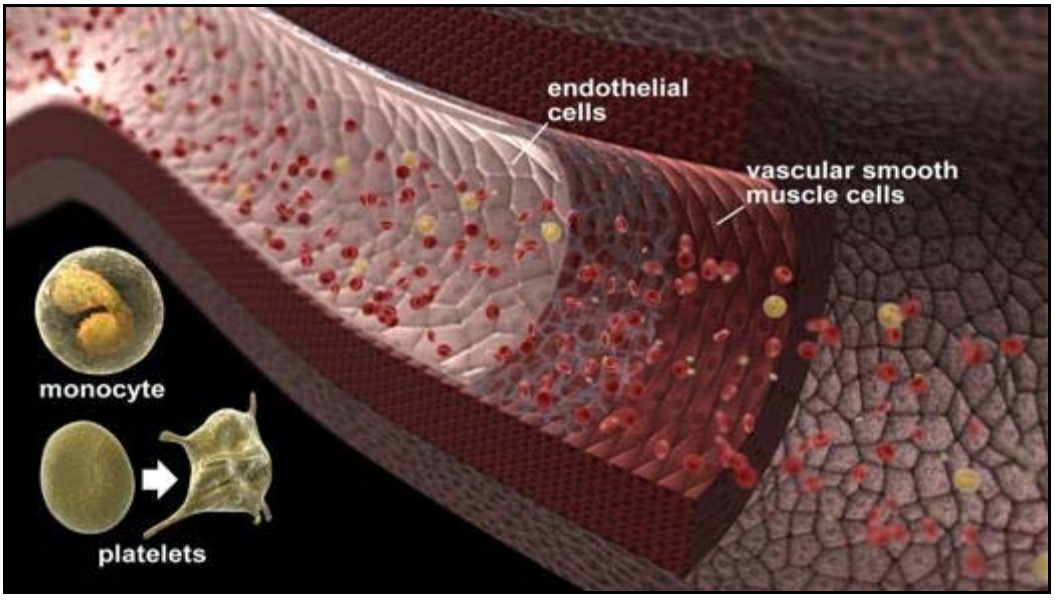

Figure 1 Key regulators of thrombosis. Platelets, vessel wall and monocytes are all involved in thrombosis.

1.1.1 Platelets and vessel wall in the thrombosis - From the first characterization of platelets in the 19th century as "small, curious beads or grains which accumulate to each other to irregular clusters", the knowledge about platelets grew very fast. At present, it is widely established that platelets are not only implicated in thrombotic but also in inflammatory reactions, immune response, in atherosclerosis and in hematogenic

metastasis (Weber, et al., 2004) (Lievens, et al., 2001) (Gawaz, et al., 2005). As a-nucleate cells with a diameter of 2-4 μm, platelets are released from megakaryocytes in the bone marrow (Jurk, et al., 2005) and show a lack of genomic DNA (Italiano, et al., 2003) but still contain megakaryocyte-derived mRNA and translational machinery needed for protein synthesis (Nieswandt, et al., 2009). The established function of platelets is to stop hemorrhage after tissue trauma or vascular injury. On the other hand, platelets form thrombus at site of ruptured atherosclerotic plaques, and in this way trigger heart attack and stroke (Jurk, et al., 2005). The immobilization of platelets at sites of vascular injury requires specific platelet-vessel wall- (adhesion) and platelet-platelet- interaction (aggregation). The ***adhesion*** of platelets to the subendothelial matrix is the initial step in primary hemostasis. Platelets interact with extracellular matrix (ECM) proteins via specific adhesive glycoproteins (GP). In contrast to resting platelets, which are discoid with homogenously distributed granules, activated platelets show a change in the assembly of cytoskeleton proteins resulting in a shape change with extensive formation of pseudopodia originating from the plasma membrane (Fox, 1993). Further, the granules centralize and fuse with the plasma membrane via exocytosis with ***secretion*** of the granule content. Some secretion products, such as adenosine diphosphate (ADP) and serotonin, potentiate the stimulation of more platelets, which are attracted to the damaged vessel wall. The activation of platelets is associated with the binding of fibrinogen (Savage, et al., 1996) to its major receptor GPIIbIIIa, which is essential for platelet bridging and subsequent ***aggregation***. Fibrinogen is thought to play an important role particularly at the low shear rates characteristic of the venous circulation. Anyway Ni et al. demonstrated, in fibrinogen- null mice, that platelet deposition and the onset of thrombus formation are the same as in WT mice (Ni, et al., 2000). However, thrombi in fibrinogen-null mice are unstable and embolize as they become larger, demonstrating that also other adhesion molecules are important for the formation of a stable clot. During secondary hemostasis, the amplification of platelet stimulation leads to ***procoagulant activity***, ***thrombin generation*** and formation of a stable platelet-fibrin plague with subsequent ***clot retraction*** (Jurk, et al., 2005). In certain conditions of flow, platelets have to slow down to stop at sites of vascular damage. The high molecular weight (1-10 MDa) multimeric plasma protein von Willebrand factor (vWF) associates with the major matrix protein collagen on the surface of the subendothelium and serves

as a substrate of platelet adhesion predominantly under high shear. Different types of collagens are present in the vessel wall, of which collagens I and III are considered the most important in supporting platelet adhesion to the damaged vasculature. Type I, III and VI collagen filaments have affinity for vWF and the two molecules are associated in the ECM. While soluble vWF does not bind to platelets to prevent aggregation in the normal circulation, immobilized vWF onto collagen is highly reactive toward flowing platelets. This may be because immobilized vWF assumes an extended shape under the effect of shear allowing its A1 domain to interact with platelets (Siedlecki, et al., 1996) (Schneider, et al., 2007). Platelet adhesion to vWF is a dynamic process in which initial platelet tethering is characterized by transient interactions mediated through GP Ibα. This deceleration then allows platelets to form new bonds with slower intrinsic binding kinetics and to become activated (Savage, et al., 1998). It has long been known that the GP Ib/IX/V complex is the major platelet receptor mediating interaction with vWF. In addition to vWF, the GP Ib/IX/V complex also binds to other adhesive proteins (collagen, thrombospondin-1), α-thrombin and coagulation factors. *In vivo* experiments on vWF-null mice display prolonged bleeding times and spontaneous bleeding in about 10% of neonates (Denis, et al., 1998). Anyway, in the complete absence of vWF, platelet accumulation on the vessel wall is significantly decreased but not absent. These results argue both for the importance of vWF in platelet deposition on the vessel wall and for vWF-independent mechanism for arterial thrombus formation. On the other hand mice expressing modified GP Ibα can not form a thrombus, demonstrating that this glycoprotein is essential for arterial thrombus formation (Bergmeier, et al., 2006).

GPVI, the major signaling collagen receptor on platelets, is a member of the immunoglobulin superfamily (Clemetson, et al., 1999). Deficiency in GPVI leads to impaired collagen-induced platelet adhesion and aggregation and is associated with a mild bleeding disorder (Moroi M, 1989).

However, the central platelet receptor in regulating platelets aggregation is the $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) that link activated platelets through fibrinogen bridges. Interaction between GPIIb/IIIa and its ligand is associated with molecular conformational changes, resulting in a firm connection. GPIIb/IIIa can bind also other ligands such as vWF, fibronectin, vitronectin and CD40L.

Integrin $\alpha_2\beta_1$ (GPIa/IIa) is a less abundant platelet integrin (Clemetson, et al., 2007). Also it binds to collagen. As platelets on immobilized GPIa/IIa show limited signaling, it appears that this integrin mainly serves to support platelet interaction via the other collagen receptors (Munnix, et al., 2008). Integrin $\alpha_v\beta_3$ (binding vitronectin and fibrinogen), integrin $\alpha_5\beta_1$ (binding fibronectin) and integrin $\alpha_6\beta_1$ (binding laminin) are moderately expressed on platelets (Clemetson, et al., 2007) and also their relevance is moderate (Gruner, et al., 2003). The adhesive molecule fibronectin, an ~500 kDa glycoprotein dimer, is abundantly present in blood plasma, megakaryocytes, and α-granules of platelets (Maurer, et al., 2010). Data from men or mice lacking fibronectin suggest that it has only a limited role in normal hemostasis. However, fibronectin levels do affect thrombus formation and thrombus stability (Maurer, et al., 2010) (Ni, et al., 2003). Vitronectin also is abundantly present in plasma and ECM, coordinates migration and signaling of blood cells and vascular cells, but its role in platelet activation is unclear (Preissner, et al., 2011). Other vitronectin ligand is PAI-1. Vitronectin binding stabilizes PAI-1 as a fibrinolytic inhibitor, which renders fibrin clot less susceptible for lysis (Zhou, et al., 2003). Two other adhesive proteins with an established role in platelet-vessel wall interaction are thrombspondin-1 and laminin. Thrombospondin-1 is released from α-granules of platelets and binds to CD36, resulting in platelet activation (Jurk, et al., 2003). Laminin is a large glycoprotein (920 kDa) of the ECM and the basement membrane, synthetized by endothelial cells, which becomes exposed after mild vascular injury (Hawiger, 2001).

Platelet activation by ECM proteins results in the release or production of several granule components that modulate functions of interacting platelets and blood and vascular cells. Several secretion products of immobilized platelets stimulate additional circulating platelets, which are recruited to form aggregates. The dense bodies of platelets contain important secondary agonists like ADP or serotonin. ADP is predicted to be prominent amplifier of initial platelet activation (Gachet, 2001). There are two important ADP receptors on the platelet surface. The $P2Y_1$-receptor mediates mobilization of Ca^{2+} and shape change and transient aggregation (Fabre, et al., 1999). The $P2Y_{12}$- receptor is believed to potentiate platelet secretion and to be involved in sustained irreversible aggregation (Dorsam, et al., 2004). Serotonin amplifies together with ADP the platelet response. In addition, serotonin may play a procoagulant role in

augmenting the retention of procoagulant proteins like fibrinogen and thrombospondin on the platelet surface (Dale, et al., 2002). The α-granules contain large adhesive proteins (vWF, thrombospondin-1, vitronectin, fibronectin), mitogenic factors (PDGF, VEGF, TGFβ), coagulation factors and protease inhibitors (protein C, PAI-1, TFPI) that are released immediately after platelet activation. Other soluble agonists of platelets are thromboxane A_2 (TXA_2) produced by the platelet cyclooxygenase/thromboxane synthase complex and thrombin formed at the platelet surface.

Indeed the formation of a stable platelet plug during secondary hemostasis is characterized by thrombin-mediated conversion of fibrinogen to fibrin. Thrombin is also generated on surfaces of blood and vascular cells. However, the platelet membrane contains a specific lipid assembly and receptors with high-affinity binding sites for clotting factors, a favored preferential and specialized locus to induce and modulate secondary hemostatic processes (Monroe, et al., 2002).

Finally, platelets carry a multitude of inflammatory mediators, such as chemokines, including the most abundant platelet chemokine, PF4 (CXCL4), which can deposit onto the vessel wall and contribute to the development of vascular lesions (Pitsilos, et al., 2003). Further, platelets shed proinflammatory microparticles during storage and upon activation, which have the potential to affect the function of the vessel wall and circulating leukocytes (Agouni, et al., 2008).

1.1.2 Coagulation system - In the 1960s, two independent groups constructed a model for coagulation that resembled a waterfall or cascade. Therefore, this model was aptly named the coagulation cascade model (Davie, et al., 1964) (Macfarlane, 1964). Herein, each clotting factor consists of a pro-enzyme that is converted to an active enzyme by the upstream activated clotting factor. It was also suggested that two different cascades exist and converge in FX activation. These are named the ***intrinsic pathway***, so called because all the components are present in the blood, and the ***extrinsic pathway*** requiring an external factor (Tissue Factor (TF) from the extravascular tissue.

The coagulation factors are usually divided into two biochemical classes: the serine protease (i.e., (pro)thrombin, FVII, FIX, FX and FXI) and serine protease cofactors (i.e., thrombomodulin, TF, FV and FVIII). The natural anticoagulant protein C also belongs

to the group of serine proteases. These proteolytic enzymes are the active components of the clotting machinery and share structural features. Full-length FVII, FIX, FX and protein C consist of an NH_2-terminal γ-carboxylated glutamic acid domain, two epidermal growth factor-like domains (EGF1 and EGF2) and a serine protease domain. Prothrombin has a similar structure but contains two kringle domains instead of the EGF domains, while FXI is a homodimer with four apple domains in each subunit.

Upon vasculature disruption sub-endothelial cell like smooth muscle cells and fibroblasts become exposed to the bloodstream and expose a key initiator of the coagulation cascade, TF, which, acting as a cofactor, binds coagulation FVII and converts 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 have adhered to a site of injury. In parallel, thrombin will convert (platelet-derived) FV into FVa, thus amplifying prothrombinase activity, and will convert 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 catalyzes the formation of covalent crosslink between adjacent fibrin chains to yield an elastic, polymerized fibrin clot (Ariens, et al., 2002) (*Figure 2*).

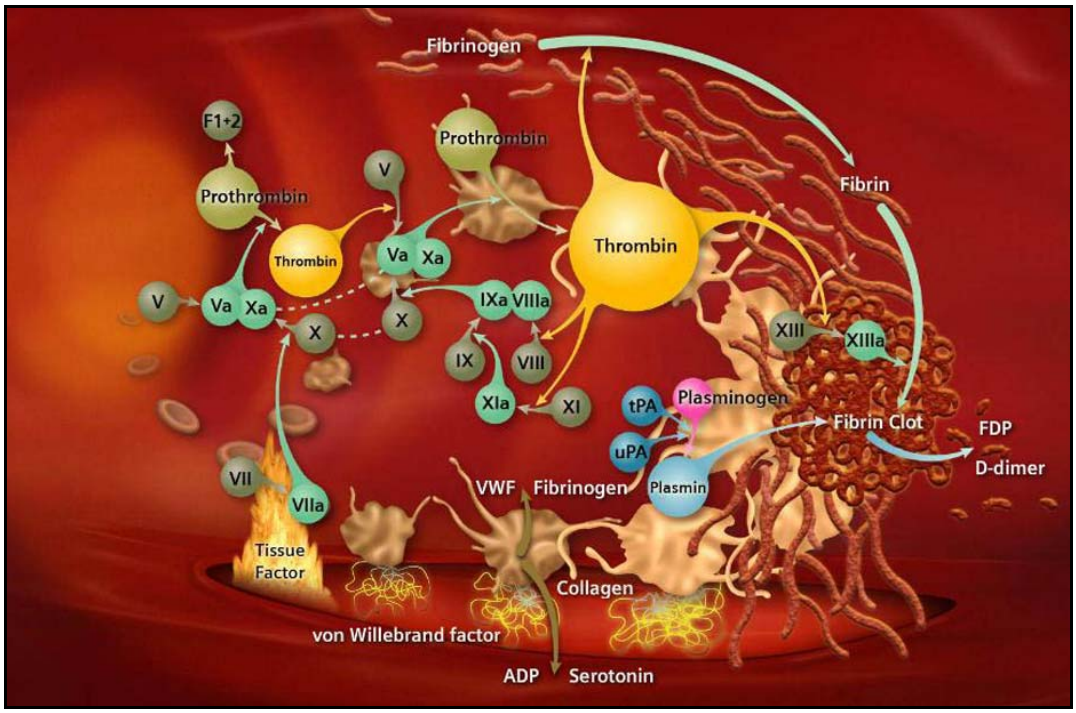

Figure 2 Coagulation cascade. The coagulation cascade comprises two different pathway: these are named the *intrinsic pathway*, so called because all the components are present in the blood, and the *extrinsic pathway* requiring an external factor (Tissue Factor (TF) from the extravascular tissue.

On the other hand, several studies have clearly shown that extensive ***negative control of coagulation*** (Broze, et al., 1988) (Jesty, 1978) is essential to prevent uncontrolled, widespread clot formation. First, circulating protease inhibitors, such as ***antithrombin***, considered one of the most important inhibitors of thrombin generation and function and acts inhibiting FIXa, FXa and thrombin, heparin cofactor II, and ***TFPI*** that inhibits coagulation by direct inhibition of free FXa and by interaction with the transient TF/FVIIa/FXa complex (Girard, et al., 1989). The second anticoagulant modality is provided by the enzyme-based ***protein C/protein S pathway*** that establishes proteolytic inactivation of FVIIIa and FVa, thus suppressing tenase and prothrombinase actions.

Tissue Factor (TF) and thrombosis. Tissue Factor (TF), formerly known as thromboplastin or CD142, is a 47-kDa transmembrane glycoprotein expressed in extravascular tissue, by fibroblasts and smooth muscle cells, serving as a hemostatic "envelope", poised to activated coagulation upon vascular injury (Versteeg, et al., 2013) (Wilcox, et al., 1989). The TF gene is located on chromosome 1 and consists of 6 exons (Mackman, et al., 1989). One main transcript as well as at least one alternatively spliced

form have been described (Bogdanov, et al., 2003). The mature TF protein comprises a 219-amino acid extracellular region, a hydrophobic transmembrane region, a short intracellular tail of 21 amino acids. The external part consists of two fibronectin-type III domains, each with an extracellular disulfide bond (Versteeg, et al., 2013). Generally, active TF is not exposed to the blood stream, but endothelial cells and adhered leukocytes may express active TF as a response to injury or to inflammatory stimuli such as endotoxin, chemokines or cytokines (Erlich, et al., 1999) (Warr, et al., 1990). For example endothelial TF is induced by cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β or CD40 ligand (Bavendiek, et al., 2002); by biogenic amines such as serotonin (Kawano, et al., 2001) or histamine (Steffel, et al., 2005), and by mediators such as thrombin, oxidized LDL or vascular endothelial growth factor (Camera, et al., 1999). In addition to its role in coagulation, TF participates in other cellular processes. It is involved in migration and proliferation of vascular smooth muscle cells (Pyo, et al., 2004) and it has also been observed to promote tumor neovascularization and metastasis (Steffel, et al., 2006).

Aberrant expression, initiates life-threatening thrombosis in a variety of diseases, including atherosclerosis, cancer and sepsis (Tremoli, et al., 1999) (Rickles, et al., 2003) (Rao, 1992). For instance, rupture of atherosclerotic plaques exposes plasma clotting factors to high levels of TF with subsequent thrombosis (Tremoli, et al., 1999). TF is also expressed by circulating monocytes and tumor cells that may initiate disseminated intravascular coagulation (Rickles, et al., 2003) (Levi, et al., 1999). Finally TF may be shed from cells into the circulation in the form of microparticles (Giesen, et al., 1999) (Marmur, et al., 1993). Microparticles are 50- to 1000-nm membranes vesicles that are released from various cells. (Freyssinet, et al., 2010). This circulating TF has been referred to as blood-born TF and may contribute to thrombosis in different diseases but it is still unclear the strength of its influence.

The level of circulating TF to thrombosis is currently a highly debated topic. Levels of TF in the vessel wall are reported to far exceed the amount in blood with an estimated ratio of 1000:1 (Butenas, et al., 2005) (Day, et al., 2005). Other investigators have reported higher concentrations of TF in the blood of healthy individuals (Diamant, et al., 2002) (Giesen, et al., 1999) (Siddiqui, et al., 2002).

Transgenic mice $mTF^{-/-}/hTF^{+}$, generated from TF deficient embryos with a minigene (hTF) containing the human TF promoter and cDNA, express very low levels of TF in

different tissues, have a prolonged tail bleeding time and are prone to excessive bleeding during major surgery compared to wild-type mice. In addition, whole blood clotting time is significantly prolonged using blood from low TF mice compared with wild-type mice (Pawlinski, et al., 2004), suggesting that low TF mice have reduced TF in both the vessel wall and the blood. Low TF mice have prolonged occlusion times compared to wild-type mice in a carotid model of thrombosis involving oxidative damage to the vessel wall. Similarly, in a heat-induced injury model of thrombosis using the microvasculature of the cremaster muscle, low TF mice have reduced TF and fibrin levels in the thrombus compared with wild-type mice (Pawlinski, et al., 2004). Bone marrow transplantation experiments showed that the occlusion time in the carotid thrombosis model was not affected by dramatically reducing TF in hematopoietic cells (Pawlinski, et al., 2004), providing evidence that cells within the vessel walls were the primary source of TF-initiated clotting in this model. In contrast, reducing hematopoietic cell TF was associated with a significant reduction in TF and fibrin levels in the model of microvascular thrombosis, suggesting that hematopoietic cells release TF positive microparticles into the circulation and that these microparticles contribute to thrombosis. Indeed, Furie and colleagues have shown that microparticles bind to activated platelets within the thrombus via an interaction between PSGL-1 and P-selectin (Falati, et al., 2003).

Platelets appear to acquire TF through interaction TF-bearing microparticles from monocytes (Del Conde, et al., 2005) (Falati, et al., 2003) (Hrachovinova, et al., 2003), but platelets also contain TF pre-mRNA (Camera, et al., 2003), which can be spliced into mature mRNA upon platelet activation, culminating in limited TF protein expression and procoagulant activity (Schwertz, et al., 2006). Because platelets secrete large quantities of the TF antagonist tissue factor pathway inhibitor (TFPI), the physiological relevance of this low expression of TF to hemostasis is unclear.

Recently Camera's group demonstrated that in acute coronary syndrome (ACS) patients more than 3 times as many circulating platelets expressing TF on their surface as are found in stable angina (SA) patients and in controls. They observed that not only is the number of TF-positive platelets higher in ACS, but each platelet express twice the number of TF molecules than in the other two groups, so that the total capacity to generate thrombin is greater. Thus, they conclude that TF-bearing platelets as well as

platelet-leukocyte aggregates (PLA) might be responsible not only for the increase in thrombus formation triggered by unstable plaque rapture, but also may provide potential distal sites for the generation of new thrombi (Brambilla, et al., 2008). Even if Camera and colleagues have not performed a direct comparison of TF activity between platelets and monocytes, and even if the TF activity of a single monocyte is higher than that of a single platelet, the number of the latter in a thrombus is largely greater compared to the number of monocytes. Moreover, also TF mRNA is increased in ACS patients. Irrespective of the mechanism involved in platelet-produced TF (derived by their progenitors megakaryocytes or by a greater state of inflammation), the higher amount of TF mRNA found in platelets from ACS patients further strengthen their prothrombotic potential, especially in view of the platelet biosynthetic potential. The platelet-derived TF may contribute to fibrin formation and to the propagation and stabilization of a thrombus but can also participate, as recently shown, in several cellular processes that stimulate atherogenesis (Servoss, et al., 2002).

1.1.3 Leukocytes and thrombotic disorders – The involvement of leukocytes in thrombus formation, first observed *in vivo* by Giulio Bizzozero in 1881 (de Gaetano, 2011), was documented in *in vitro* experimental studies and in animal models (Wagner, 2005) (Cerletti, et al., 2010) (Cerletti, et al., 1999); however, the mechanisms of leukocytes involvement in thrombosis and their modulation are not completely clear. Most likely, leukocytes, besides a direct inflammatory effect, may interact with platelets, form mixed aggregates and participate in a final common thrombogenic effect (Cerletti, et al., 2012).

The adhesion of leukocytes to platelets, deposited at the site of vascular injury, may represent an important mechanism by which leukocytes contribute to hemostasis and thrombosis (Harding, et al., 2007).

Through transient and stable interaction with the endothelium, platelets may induce and propagate a chronic inflammation of the vessel wall, resulting in the development and progression of atherosclerotic lesions (Gawaz, et al., 2005). Under these inflammatory conditions, platelets show robust interaction with leukocytes (Van Gils, et al., 2009). This binding may occur in circulation or upon the adherence of platelets to the vascular wall, thereby providing an adherent surface to recruit leukocytes (Gawaz, et al., 2005) (Weber, et al., 1997). Leukocytes initially adhere to platelets via PSGL-1-P-selectin interactions

(Evangelista, et al., 1999) (Yang, et al., 1999) and this adhesion is subsequently stabilized through binding of Mac-1 to the GPIbα (Simon, et al., 2000). Engagement of PSGL-1 and Mac-1 induces an inflammatory cascade in monocytes, causing them to secrete effector molecules such as CCL5 (Weyrich, et al., 1996) (Neumann, et al., 1997). Lymphocytes have also been shown to interact with platelets, in a P-selectin-, CD154- and $\alpha_{IIb}\beta_3$-dependent manner (Hu, et al., 2010). Also thrombospondin is reported to be involved in the cross-link between platelets and monocytes in early vascular injury via an interaction with GP IV on the surface of both cells (Silverstein, et al., 1989). The binding of platelets to leukocytes facilitates the interaction of the resulting platelet-leukocytes complexes to endothelial cells (Kirton, et al., 2000) (Kuckleburg, et al., 2011), providing a possible mechanism for the accelerated progression of atherosclerosis observed in mice challenged with activated platelets (Huo, et al., 2003) (Lievens, et al., 2010). Also, in patients with cardiovascular disease, platelet neutrophil and platelet monocyte complexes were shown to be elevated and presented a marker for acute MI (Ott, et al., 1996) (Furman, et al., 2001). Thus, platelet-leukocyte aggregation might not only be a byproduct of local platelet activation at the site of an unstable or ruptured plaque but might also play an integral role in the pathogenesis of atherosclerosis and MI.

However, the main physiological role of monocytes is attributed to innate immunity and the development of tissue macrophages and dendritic cells, but pathophysiological role of these, goes far behind these limits. Firstly, monocytes appear to be the major source of blood TF. Monocytes expose considerable quantities of TF on their surface being stimulated by a variety of inducers of which lipopolysaccharides (LPS) and C-reactive protein are especially important (Cermak, et al., 1993) (van der Logt, et al., 1994). The amount of TF mRNA performed by resting monocytes does not significantly affect the surface density of this protein but nonetheless, this appears to be responsible for the substantial variation of surface TF expression following stimulation by LPS (Sovershaev, et al., 2007). Monocytes transmigration through the arterial wall to tissues prior differentiation into macrophages is also associated with enhanced TF generation (McGilvray, et al., 2002). Moreover, monocytes stimulate TF expression by endothelial cells. Additionally, given that megakaryocytes do not express TF but the factor is present on platelets in circulation it is possible that TF-bearing monocytes-derived microparticles may be

involved in transfer of TF to platelets in addition to the production of the factor from stored intra-platelet mRNA (Panes, et al., 2007) (Scholz, et al., 2002).

Multiple factors seen in coronary artery disease (CAD) promote excessive monocyte TF formation, that observation accords with the prothrombotic state typical for the disease. Indeed, extracts of human atherosclerotic plaque significantly enhance monocyte TF expression (Lambert, et al., 2007) (Muhlfelder, et al., 1999).

Participation of monocytes in the prothrombotic conditions is not restricted to the expression of TF and generation of monocytes-platelet aggregates (MPA). The evidence support the impact of monocytes for thrombus generation, both directly via the secretion of procoagulant factors and indirectly, by promoting inflammation processes (Shantsila, et al., 2009). It is well known that inflammation affects the activity of various coagulation pathways. The inflammatory state also activates monocytes, which possess receptors for CRP and express CD40, a receptor for CD40 ligand (Bharadwaj, et al., 1999) (Crowell, et al., 1991) (Stumpf, et al., 2003). CRP and CD40 ligand induce TF expression in human monocytes, thus linking inflammation, coagulation and thrombosis (Penn, et al., 2001) (Pepys, et al., 2001). Stimulation of human monocytes/macrophages through CD40 further induces the expression of interstitial collagenase (MMP-1), stromelysin (MMP-3), recognized elements of plaque destabilization and predictors of atherothrombotic events (Mach, et al., 1997). Activated monocytes are able to bind and activate coagulation factor X responsible for the conversion of prothrombin to thrombin. Additionally, monocytes can bind coagulation factor V and also produces its activated form on their surface (Kappelmayer, et al., 1993). Finally, to counterbalance their prothrombotic activity, resting monocytes produce thrombomodulin (Satta, et al., 1997), that is an essential cofactor of thrombin in the triggering of the natural anticoagulant protein C pathway, as explained previously.

1.1.4 Fibrinolytic process in thrombosis regulation – Beside negative modulator of coagulation activation, the ***fibrinolytic system*** is another way to control hemostasis and subsequently to prevent thrombosis. Thrombolytic agents are plasminogen activators that convert the zymogen plasminogen to the active enzyme plasmin, which degrades fibrin and activates matrix metalloproteinases (MMPs) that, in turn, degrade extracellular matrix (Collen, et al., 1991) (Birkedal-Hansen, 1995). Two physiological

plasminogen activators (PAs) have been identified: tissue type PA (tPA) and urokinase-type PA (uPA), which binds to a cellular u-PA receptor (u-PAR). Inhibition of the plasminogen/MMP system occurs at the level of the PA, by specific PA inhibitors (PAIs), at the level of plasmin, by α_2-antiplasmin, or at the level of MMPs. The two most important PAIs are PAI-1 and PAI-2. A deficient fibrinolytic response may be caused by impaired release of t-PA from the vessel wall or by increased rate of neutralization (Lijnen, et al., 1989). The PAI-1 concentration in plasma is increased in several diseases, including venous thromboembolism, obesity, sepsis, and CAD. On the other hand, increased levels of tPA or deficiency of α_2-antiplasmin or PAI-1 may cause a bleeding tendency (Collen, 1999).

2 Relationship between CVD and emerging risk factors: the depression

Some reviews about the link among depression and CVD have concluded that epidemiologic evidence supports "the notion that mood disorders confer risk for coronary disease, but it is premature to describe it as a causative factor" (Barrick, 1999). Interestingly, as we learn more about the expression of emotions, it appears these perceptions may simply be the language representation of the somatic feelings (Ranga, et al., 2003). In fact, sadness is often portrayed as feeling of heaviness in the chest or as a "broken heart". Formerly, premature conclusions about a causal relationship for depression leading to coronary disease, resulting in gaps in the literature on the nature and duration of depression, the biological mechanisms linking these two disorders, and the effect of antidepressants treatment on the risk for coronary disease were drawn (Appels, 1997). On the other hand, recently, some studies have demonstrated that patients with depression have an increased risk of developing cardiovascular disease and a 2- to 4-folds risk of mortality after experiencing a cardiac event (Penninx, et al., 2011). Despite the evidence that heart disease and depression are epidemiologically linked, this correlation is not well understood

When considering the co-morbidity between major depressive disorders (MDD) and CVD it is important to differentiate between ***a***) the prevalence and impact of MDD in those existing CVDs and ***b***) MDD as a risk factor for the development of CVD.

Evidence in support of the relationship between depression and cardiovascular disease derives from growing array of cross-sectional surveys demonstrating the disproportionately elevated rates of depression among people with documented CVD compared with matched community-based samples. About a quarter of patients with angiographically demonstrated coronary atherosclerosis, recorded mild depressive symptoms and a further 11% had moderate to severe depression (Barefoot, et al., 1996). Importantly, the presence of depression in patients with CVD has been convincingly demonstrated to increase mortality (Frasure-Smith, et al., 1995) (Barefoot, et al., 1996) (Carney, et al., 1988). Frasure-Smith and Lesperance (Frasure-Smith, et al., 2008) recently extended this observation, documenting that depression and generalized anxiety disorder predict greater risk of major adverse cardiac events in the 2 years following MI.

The association between mental and cardiovascular health was noted early in the 20th century when Malzberg (Malzberg, 1937) reported high mortality rates in hospital in patients with melancholia. It was not until 50 years later that evidence from prospective trials indicated that affective disorders participated in the development of CVD. This elevated risk is independent of classical risk factors such as smoking, obesity, hypercholesterolemia, diabetes mellitus and hypertension. In addition, in a recent meta-analysis on the significance of depression in contributing to CVD development, emerges that depression at baseline is associated with the increased risk of development of a wide range of cardiovascular outcomes including MI, fatal and nonfatal heart disease and stroke (Van der Kooy, et al., 2007).

In contrast, the frequency of developing depression after a cardiac event seems to be irrelevant. In fact, although 50% of patients immediately following MI are affected by mood disorders (Cassem, et al., 1977), the risk of developing depression is highest among patients who had prior episodes of depression (Lloyd, et al., 1983). Moreover, only 18% of 50 patients with documented CAD show major depressive symptoms (Carney, et al., 1987). Thus, depressive symptoms are common, but full-blown major depression is seen only in 20% of patients (Ranga, et al., 2003).

Therefore, it was formulated the hypothesis that depression is cause, not consequence, of cardiovascular events.

Indeed, depression increases the risk of dying among patients who have just had an MI (observed in depressed patients) (Stern, et al., 1977), and that mental stress could provoke

ischemia (Jiang, et al., 1997). Patients with established CAD and depressive symptoms showed more ischemia during mental stress testing (Jiang, et al., 1997), suggesting that even emotions within the normal range can play a key role in this relation.

Patients with depression have been found to have elevated plasma norepinephrine, increased heart rates and reduced heart-rate variability. Reduced heart-rate variability has been associated with increased mortality in both CAD and chronic heart-failure (Jiang, et al., 1997) (Kleiger, et al., 1987), supporting this second hypothesis about the relationship between depression and cardiovascular disease. In addition, the depression reduces motivation and individuals do not care of their general health: depressed patients exercise less (Siegler, et al., 1997) (Adler, et al., 1994), eat poorly, smoke more and in general exhibit behaviors that increase the risk for cardiac disease.

However, recently the existence of a common cause underlying both the depression and cardiovascular disease (Miller, et al., 2000) was hypothesized. In fact, the depression promotes a mild inflammatory response by fostering maladaptive health practices (cigarette smoking, sedentary lifestyle), triggering dysregulation of hormonal system (hypothalamic-pituitary-adrenocortical and sympathetic-adrenal-medullary axes) and increasing susceptibility to infections associated with atherosclerosis (Carney, et al., 2002). Over time this inflammatory response contributes to CVD progression by facilitating the growth of atherosclerotic plaque, precipitating the rupture of established plaque, and potentiating coagulation processes involved in thrombogenesis (Ross, 1999) (Libby, 2001).

2.1 Potential mechanisms linking cardiovascular disease and depression

– While the mechanisms linking depression and cardiovascular disease are not well understood, a number of relationships have been identified which demonstrate the plausibility and coherence of a causal relationship. As the etiology of major depression, linked to brain monoaminergic neuronal dysfunction (Schildkraut, 1965) (Maas, 1975) (Linnoila, et al., 1982), alterations in monoamine receptor sensitivity (Charney, et al., 1981) and stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis (Dinan, 1994) with concomitant reduction in brain neurotrophins (Duman, et al., 1997). Several hypothesis were formulated. These include both direct biological mechanisms and indirect pathways mediated through behavioral, lifestyle and social factors (Goldston, et al., 2008).

2.1.1 Behavioral mechanisms - A number of behavioral pathways appear to be involved in the relationship between psychological risk factors and CVD. Patients who are depressed have been found to be less likely to adopt a healthy lifestyle to reduce their risk of developing cardiovascular disease or to reduce the risk of disease progression once CVD is established (Carney, et al., 1987). Depressed patients have also been shown to be less likely to undertake physical activity (Paffenbarger, et al., 1994) and are less compliant with dietary restriction (Lesperance, et al., 1996). In addition, smoking rates are higher in depressed people and they also find it more difficult to quit (Anda, et al., 1990) (Glassman, et al., 1990).

2.1.1.1 Adherence to treatment - A recent meta-analysis examining the impact of depression on treatment adherence, found that patients with depression were three times more likely to be non-adherent with treatment regimens (DiMatteo, et al., 2000). Cardiac patients with depression have also been shown to be less likely to follow recommendations to reduce ongoing cardiac risk factors following MI (Goldston, et al., 2008).

2.1.1.2 Social support - A strong and consistent inverse relationship has been found between the magnitude of social support and both the development of CVD in initially healthy people and adverse prognostic outcome in those with existing CVD (Bunker, et al., 2003). Lack of both structural and functional social support has been implicated in the poorer prognosis in depressed cardiac patients. Factors such as living alone (Case, et al., 1992), being socially isolated (Ruberman, et al., 1984), lack of available support (Gorkin, et al., 1993), low perceived social support (Frasure-Smith, et al., 2000), lack of a close confidant (Williams, et al., 1992) and low emotional support (Berkman, et al., 1992) have all been found to increase morbidity and mortality.

2.1.2 Biological mechanisms

2.1.2.1 Dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis - Depression has been associated with stress-induced activation of the hypothalamic-

pituitary-adrenal (HPA) axis (Dinan, 1994), resulting in over stimulation of the sympathetic nervous system, which in turn increases circulating catecholamines (adrenaline and noradrenaline) and serum cortisol (Banki, et al., 1992) (Brown, et al., 2004). Hypercorticolaemia causes blunted HPA activity and diminished feedback control (Carroll, et al., 1981). This neuro-hormonal abnormalities lead to an imbalance in sympathetic and parasympathetic activity, resulting in surges in heart rate and blood pressure and increasing the risk of atherosclerotic plaque rapture and acute coronary thrombosis. High levels of circulating cathecolamines also increase the irritability of the heart muscle, causing ventricular arrhythmias, which may lead to ventricular fibrillation and cardiac arrest (Carney, et al., 1999). Moreover the physiological consequences of increased circulating cortisol are diverse and include importantly, in the context of cardiac risk, the onset of metabolic changes, such as insulin resistance, hyperinsulinaemia, glucose intolerance and hyperlipaemia, and increased visceral fat mass (Posener, et al., 2000).

2.1.2.2 Disturbance in cardiac autonomic tone - One of the mechanisms by which depression increases the risk of mortality in patients following MI is the adverse impact that depression has on cardiac autonomic tone and heart rate variability. Ventricular arrhythmias are responsible for most cases of sudden cardiac death in the post-MI period and are increased in patients with depressive co-morbidity (Carney, et al., 1999) (Gorman, et al., 2000). Decreased heart rate variability (HRV) increases the risk of ventricular arrhythmias and has recently been demonstrated in people with depression (Stein, et al., 2000). Moreover, when depression occurs following MI, the heart rate variability is further decreased, increasing the mortality risk in depressed patients with CVD even more (Carney, et al., 2001) (Pitzalis, et al., 2001).

2.1.2.3 Disturbances in blood clotting mechanisms - Increased platelet activation and thrombus formation is an important pathogenic feature in acute coronary syndromes (MI and unstable angina) with anti-platelet therapy being one of the contemporary mainstays of therapy (National Heart Foundation of Australia and the Cardiac Society of Australia and New Zealand, 2000). Substantial disturbance in platelet function occurs in depression causing vascular damage, thrombus formation and increasing the risk of future coronary

events. Studies have demonstrated that platelet activation is increased in depressed individuals regardless of whether they have CVD (Laghrissi-Thode, et al., 1997) (Musselman, et al., 1996). More recently, evidence have revealed that depression is associated with hyperactive 5-hydroxytryptamine (5-HT) transporter$_{2A}$ receptor signal transduction system and increased responsiveness of platelets to serotonin (Schins, et al., 2003). Musselman et al. (Musselman, et al., 2000) reported that treatment with the selective-serotonin-receptor reuptake-inhibitor (SSRIs), class of antidepressant, reduced the level of platelet activity in depressed patients to normal limits. Actually, depression increases platelet aggregation (Patrono, et al., 1997). Depressed ischemic heart disease patients showed elevated β-thromboglobulin levels, increased plasma levels of platelet factor 4 and increased expression of the platelet surface receptors for glycoprotein IIb/IIIa and P-selectin compared with non-depressed subjects (Laghrissi-Thode, et al., 1997). Finally it has been demonstrated a strong positive correlation of depression with high fibrinogen, PAI-1 and tPA-antigen suggesting an involvement of also fibrinolytic processes (Matthews, et al., 2007). All these factors could play a mediating role on the effect of depression in the development of CVD (Ranga, et al., 2003).

2.1.2.4 Vascular endothelial dysfunction of the coronary arteries - Impaired vascular endothelial function is a hallmark of CVD has been found to occur in a variety of cohorts of depressed patients, including young and otherwise healthy individuals (Broadley, et al., 2002) (Rajagopalan, et al., 2001). Indeed, the role of inflammation in the pathogenesis of CVD is widely accepted. Low-grade chronic inflammation is predictive of MI and ischemic stroke. Endothelial dysfunction is a "critical intermediate phenotype" in the relationship between low-grade inflammation and CVD (Cleland, et al., 2000). Several studies have reported on the consistent presence of a low-grade chronic inflammation in depression exemplified by mild to moderate elevations in circulating biomarkers, such as high-sensitivity CRP (hsCRP) and pro-inflammatory cytokines (Piletz, et al., 2009) (Dinan, 2008) (Dantzer, et al., 2008) (Leonard, 2010). Nitric oxide (NO) is the principal mediator of smooth muscle cell relaxation and contributes to overall vascular health. Its property as a vasodilator is well established. As levels of CRP increase, basal NO generation decreases significantly (Cleland, et al., 2000). Similarly TNF-α reduces the half-life of mRNA encoding NO synthase (Yoshizumi, et al., 1991) (Halaris, 2013) resulting in

decreased basal NO production. This in turn favors increased expression of cell-surface adhesion molecules for leukocytes and platelets inducing a pro-coagulant state. In summary, endothelial dysfunction is a crucial factor in the complex relationship between depression, low-grade chronic inflammation and CVD.

2.1.2.5 Immune system activation - An increased sub-acute inflammatory immune response has been associated with depression and this may contribute to coronary artery atherosclerosis (Musselman, et al., 2000). Depressed patients have been shown to have elevated levels of inflammatory markers such as interleukin-6 (IL-6), C-reactive protein (CRP), tumor necrosis factor alpha (TNFα), as well as shifts in the relative distribution of T and B lymphocytes (Miller, et al., 2000) (Suazrez, et al., 2003). These same markers are found in inflammatory reactions associated with unstable coronary artery plaques, which manifest as acute coronary syndromes. Hence, depression may either be a cause or an effect of the same inflammatory processes that cause CVD (Anisman, et al., 2002). If depression is the cause of the inflammatory process responsible for CVD, treatment of depression should reverse or at least reduce the inflammatory component underlying atherosclerosis.

2.1.2.6 Neurotrophins – In addition to the previous mechanisms, neurotrophins may also play an important role in the connection between depression and cardiac outcomes. Depression has been strongly and consistently linked to low levels of BDNF (Hashimoto, 2010) (Duman, et al., 1997) in the hippocampal neurons, and it is thought that BDNF signaling mediates the hippocampal neurogenesis that has been linked to depression recovery (Castren, et al., 2010). Moreover, two recent meta-analysis revealed that not only central but also BDNF serum levels are reduced in MDD patients compared with healthy controls and return to levels of the control group after antidepressant therapy (Brunoni, et al., 2008) (Sen, et al., 2008). BDNF plays also a key role in several physiologic processes important to cardiovascular health. Indeed, BDNF promotes angiogenesis and survival of endothelial cells. Moreover, in pathological conditions, i.e. during hypoxia, its expression is increased (Kim, et al., 2004). Finally, BDNF may be an important mediator of the previous noted HPA axis effects on depression and cardiovascular disease. The glucocorticoid receptor interacts with specific receptor of BDNF, TrkB and

excessive glucocorticoid interferes with BDNF signaling (Kunugi, et al., 2010); therefore excess glucocorticoids may be associated with adverse outcomes via BDNF-mediated effects on endothelial cells.

3 The neurotrophins

The neurotrophins (NTs) comprise a family of at least four structurally related proteins-nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/neurotrophin-5 (NT-4/NT-5). The first member of the neurotrophin family, NGF, was discovered in the early 1950s as a target-derived protein that promotes the survival and growth of sympathetic and sensory neurons during development (Cohen, et al., 1954) (McAllister, 1999). The establishment of the NT family came with the purification and characterization of BDNF from the pig brain (Barde, et al., 1982).

Since then, two other NTs have been identified in the mammalian brain: NT-3 and NT-4 (Leibrock, et al., 1989). The genes encoding for these four NTs (*ngf, bdnf, nt-3 and nt-4*) produce 30- to 35-kDa pro-proteins; consisting of a preprodomain, a prodomain and a mature domain that are proteolytically cleaved to produce mature protein with a size of ~13-kD (Barde, 1990) (Ibanez, 1998). Until recently, little consideration was paid to the NTs prodomain, which was thought to be only involved in protein folding and in regulation of NTs secretion (Suter, et al., 1991). The pro-NTs are greatly secreted in different tissues (Bruno, et al., 2006) (Fahnestock, et al., 2001) and have biological actions, different from those elicited by mature NTs (Lee, et al., 2001). Prodomain regions have highly conserved sequences across species and modifications on prodomain change the efficient trafficking of pro-NTs from the endoplasmic reticulum. The trafficking from the endoplasmic reticulum allows the majority of pro-NTs to undergo intracellular cleavage by furin-like proprotein convertases at the consensus site R-X-K/R-R (arginine-unspecified aminoacid-lysine/arginine-arginine). The mature proteins form noncovalent symmetrical homodimers and are secreted into the extracellular environment (Chao, et al., 2002) (Mowla, et al., 2001). In addition, also pro-NTs can be secreted from the cell and processed to mature NTs (Lee, et al., 2001). With the exception of NT-4/5, NTs are highly conserved from fishes to mammals (Hallbook, et al., 1991), thus sharing a similar molecular mass (13,2-15,9 kDa), very basic isoelectric points, a somewhat unusual property for

secreted proteins, which may serve the purpose of limiting their range of action (McDonald, et al., 1991); and 50% identity in primary structure (Mowla, et al., 2001). The structural hallmarks of NTs is a characteristic arrangement of the disulfide bridges, known as cysteine knot, also identified in other secreted proteins (McDonald, et al., 1993).
The main actions mediated by the neurotrophins are the survival (McAllister, 1999), but also the differentiation (Waterhouse, et al., 2012) (Douglas-Escobar, et al., 2012), growth of neuronal processes (Bibel, et al., 2000), synaptic plasticity (McAllister, 1999) and apoptosis of neurons (Bibel, et al., 2000).

3.1 Neurotrophins sorting, secretion and cleavage - After synthesis in the endoplasmic reticulum, (pro)neurotrophins need to be folded correctly, sorted into the *constitutive* or *regulated secretory pathway*, and transported to the appropriate subcellular compartment. Many non-neuronal cells, such as smooth muscle cells, fibroblast and astrocytes, may not express molecular components of the regulated secretory pathway and, therefore, secrete neurotrophins only constitutively. Regulated secretion is prevalent in neurons. Then, neurotrophins-containing secretory granules are transported to dendrites and spines, and are secreted postsynaptically. On the other hand, neurotrophin-containing large dense core vesicles undergo anterograde transport to axonal terminals. Some studies on BDNF secretion mechanisms have highlighted that the binary decision of sorting depends on both mature and pro-domain of neurotrophins. In fact, modeling and binding studies point to a critical interaction between the acidic residues in the BDNF motif and two basic residues in the sorting receptor carboxypepatidase E (CPE) (Cool, et al., 1995). Deletion of the *Cpe* gene in mice eliminate the activity-dependent secretion of BDNF from hippocampal neurons. In addition, the results of recent experiments indicate that the interaction of the pro-domain of BDNF with sortilin, a receptor that is localized mainly intracellularly (Petersen, et al., 1997) (Nielsen, et al., 2001), controls the mode of BDNF secretion (*Figure 3*) (Chen, et al., 2005). Sortilin is co-localized with BDNF in secretory granules of neurons, and interacts with two sub-regions, ‘box2’ and ‘box 3’, both of which are in the pro-domain of BDNF. When a truncated dominant negative form of sortilin (tSort) is expressed, there is a significant decrease in the co-localization of BDNF and the secretory granule marker,

and in the regulated secretion (Chen, et al., 2005). These results indicate that in the absence of this motif, BDNF is sorted into the constitutive pathway.

Like other secreted proteins, neurotrophins arise from precursor, proneurotrophins (30-35 kDa), which are proteolytically cleaved to produce the mature proteins (12-13 kDa) (Seidah, et al., 1996). There are three ultimate fates of intracellular proneurotrophins: intracellular cleavage followed by secretion; secretion followed by extracellular cleavage; or secretion without subsequent cleavage. In particular, the 32-kDa proBDNF is the main form secreted from endothelial cells (Lee, et al., 2001), and culture neurons (Chen, et al., 2004) (Mowla, et al., 2001) (Mowla, et al., 1999). These findings indicate that mature BDNF is derived primarly from the cleavage of proBDNF by extracellular proteases, and that if uncleaved, the extracellular proBDNF might serve as an endogenous ligand. In addition, several matrix metalloproteinases (MMP), including MMP3 and MMP7, have been shown to cleave proNGF and proBDNF (Lee, et al., 2001). However the most significant protease that cleaves proneurotrophins is the serine protease plasmin (Lee, et al., 2001) (Pang, et al., 2004).

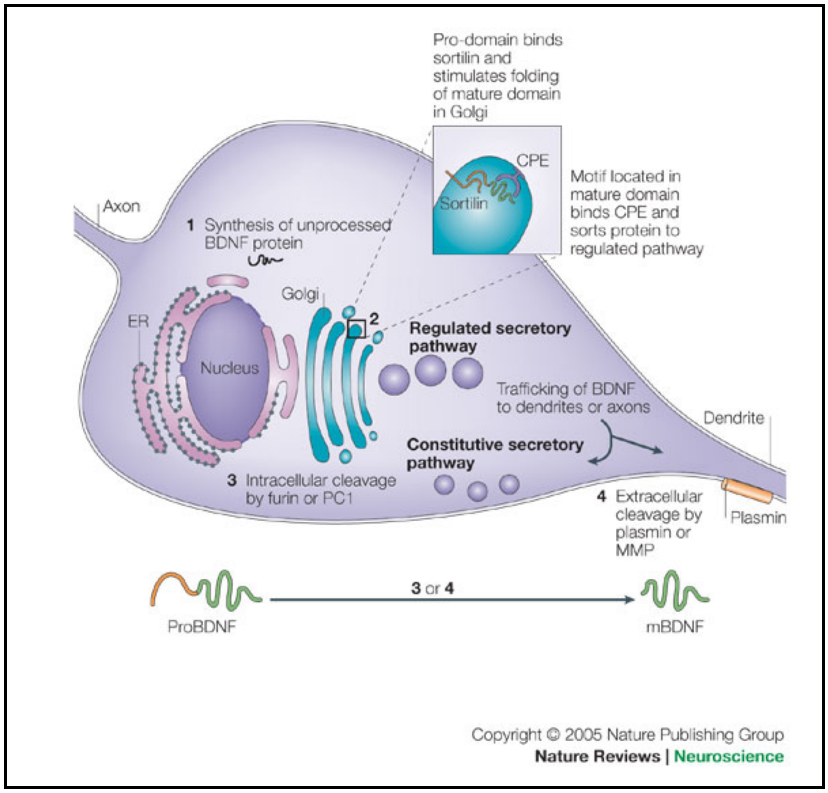

Figure 3 Example of neurotrophin sorting and releasing. First synthetized in the endoplasmic reticulum (ER), proBDNF (precursor of BDNF) binds to intracellular sortilin in the Golgi to facilitate proper folding of the mature domain. A motif in the mature domain of BDNF bind to carboxypeptidase E (CPE), an interaction that sorts BDNF into large dense core vesicles. In the absence of this motif, BDNF is sorted into the constitutive pathway. After the binary decision of sorting, BDNF is transported to the appropriate site of release, either in dendrites or in axons. Because, in some cases, the pro-domain is not cleaved intacellularly by furin or protein convertases, proBDNF can be released by neurons. Extracellular proteases, such as metalloproteinases and plasmin, can subsequently cleave the pro-region to yeld mature BDNF (mBDNF).

3.2 Neurotrophins receptors - Neurotrophins exert their effects through two classes of transmembrane receptor proteins: high affinity tyrosine kinase receptors (TrK receptors) and a lower-affinity receptor, the p75 neurotrophin receptor (p75NTR) (*Figure 4*).

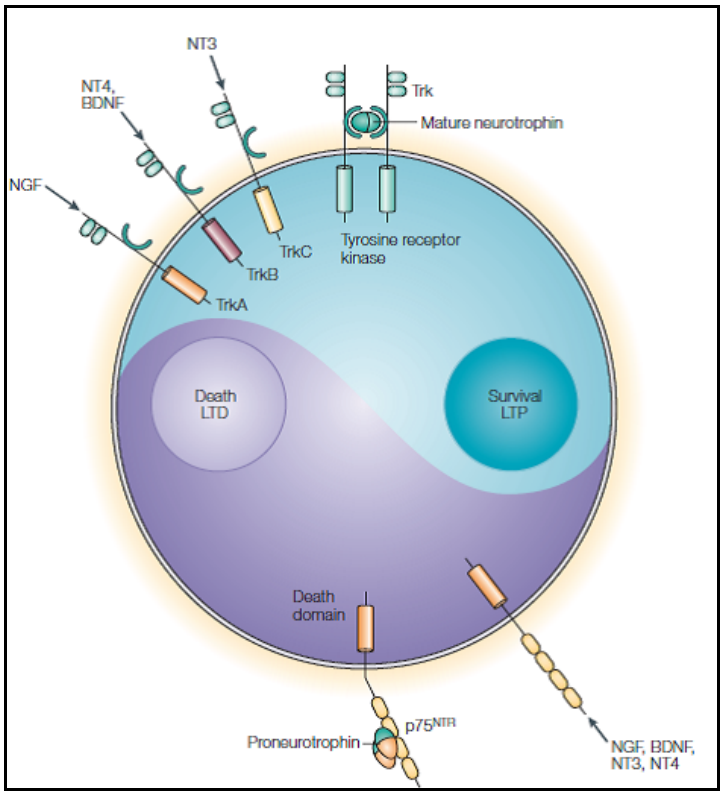

Figure 4 The actions of neurotrophins are mediated by two principal transmembrane-receptor signaling systems. Each neurotrophin receptor- TrkA, TrkB, TrkC and the p75 neurotrophin receptor (p75 NTR)- is characterized by specific affinities for the neurotrophin.

Trk receptor family - The Trk family of receptor tyrosine kinases, identified in mammals, derives its name from the oncogene that resulted in its discovery (Huang, et al., 2003). This oncogene was isolated in gene transfer assays from a carcinoma and, when cloned, was found to consist of the first seven of eight exons of non-muscle tropomyosin fused to the transmembrane and cytoplasmic domains of a novel tyrosine kinase.

The TrkA protooncogene was first identified as a NGF receptor, followed by TrkB and TrkC. NGF is the preferred ligand for TrkA, BDNF and NT-4/5 are preferred for TrkB and NT-3 for TrkC. Trk receptors share a common structural organization of their extracellular domains that clearly distinguishes them from other receptor tyrosine kinases (Schneider, et al., 1991). Immediately following the cleaved signal sequence there is an array of three leucine-rich 24-residues motifs flanked by two cysteine clusters. Two C2-type immunoglobulin-like domains are adjacent to these structures, which are followed by a single transmembrane domain and a cytoplasmic domain that contains a

tyrosine kinase domain plus several tyrosine-containing motifs similar to those present in other receptor tyrosine kinases. Like other receptor tyrosine kinases, phosphorylation of cytoplasmic tyrosines in Trk receptors regulates tyrosine kinase activity and provides phosphorylation-dependent recruitment sites for adaptor molecules and enzymes that mediate initiation of intracellular signaling cascades (Huang, et al., 2001) (Bibel, et al., 2000) (Patapoutian, et al., 2001) (Sofroniew, et al., 2001) (Kaplan, et al., 2000). The N-termini of neurotrophins are important in controlling specificity, and the structure of this region is reorganized upon binding to a Trk receptor. Interactions with Trk receptors also modify neurotrophin structures in other regions. This deformability appears important for permitting some neurotrophins to activate more than one type of Trk receptor (Huang, et al., 2003).

Splice variants of Trk receptors - Splice variants have been described for all three Trk receptors. These receptor molecules have either deletions in the extracellular domain or intracellular truncations including the tyrosine kinase domain. Insert variants of TrkA and TrkB were found to influence ligand specificity. However, their biological roles remain unclear. The truncated TrkB receptor isoforms, designated as T1 and T2, contain short intracellular domains (Klein, et al., 1990). They are up-regulated during early postnatal development and predominate over full-length TrkB in the adult brain (Fryer, et al., 1996). These truncated receptors are often expressed in non-neuronal cells that do not express full-length TrkB. (Klein, et al., 1990). They internalize BDNF and may restrict its availability (Biffo, et al., 1995).

P75NTR receptor - P75 was the first member to be molecularly cloned of a large family of receptors, which includes both TNF receptors, Fas, CD40 and ~15 other members. The defining motifs of this receptor family are cysteine repeats in the extracellular domain, which form the ligand-binding domain. None of these receptors exhibits any intrinsic catalytic activity, and they signal by associating with, or dissociating from, cytoplasmic interactors. Indeed, there are several protein association motifs in the cytoplasmic domain of p75. It is distantly related to the so-called death domain of other members of the family. All neurotrophins bind to p75 with an affinity of ~10^{-9} M, that is a lower affinity then that determined for neurotrophin binding on neurons (typically 10^{-}

^{11} M). In addition to the major transcript encoding the full-length receptor, a minor transcript exist encoding a protein truncated in the extracellular domain that has lost its ability to bind neurotrophins (Dechant, et al., 1997). Anyway about 40% of the mutant animals die during embryogenesis, apparently from defects in the formation of blood vessels (Bibel, et al., 2000). Like several other members of its family, p75 is processed by proteolysis, and relatively high levels of a soluble form of p75 have been measured in various body fluids (Zupan, et al., 1989).

3.3 Neurotrophins signaling - Binding of the neurotrophins to the Trk receptors leads to receptors tyrosine phosphorylation. Ligand-induced dimerization results in the phosphorylation of specific tyrosine residues, located in the so-called activation loop of the tyrosine kinase domain. Phosphorylation of these residues leads to an open conformation of the receptor resulting in trans-phosphorylation and allowing the access of substrates to the kinase. Phosphotyrosine residues on Trk receptors then act as docking sites for adapter molecules (Bibel, et al., 2000). Three main signaling cascades are activated by the Trk receptors and their substrates. First the activation of Ras/Raf/MEK/MAPK pathway. Inhibition of Ras activity, decreases survival of most, but not all, populations of sympathetic neurons (Borasio, et al., 1993) (Nobes, et al., 1995). Second, the activation of PI3K, implicated in neuronal survival via the activation of PKB/AKT kinase. Third, the association of PLC-γ with Trk, that regulates intracellular Ca^{2+} levels and protein kinase C activity via cleavage of the substrate PIP2 to DAG and IP3, pathway that seems important in neurotrophin-mediated neurotrophin release and in synaptic plasticity (Bibel, et al., 2000).

Whereas it is now clear that p75 transduces signals following neurotrophin binding, the function of this receptor varies considerably depending on the cellular context in which it is expressed. The first indication for the signaling function of p75 was the finding that p75 mediates sphingomyelin hydrolysis and production of ceramide following neurotrophin binding (Dobrowsky, et al., 1994) (Dobrowsky, et al., 1995). p75 seems to interact with caveolin. Caveolae are presumably the site of neurotrophin-induced sphingomyelin hydrolysis. Ceramide production is known to follow TNF binding to its receptor and to lead to NF-kB activation. Likewise, in Schwann cells expressing p75, but not catalytic

Trk receptors, NF-kB activation was observed following the addition of NGF (Carter, et al., 1996). It appears that activation of NF-kB prevents cell death. (Liu, et al., 1996)

Beyond the activation of NF-kB that seems to mediate cell survival, there are also clear indications that p75 activation by neurotrophins causes programmed cell death. Indeed, the original finding that p75 could mediate neuronal apoptosis in a neural cell line (Rabizadeh, et al., 1993) has, over the past several years, been extended to a large number of primary neural cells. Still controversial, however, is the role of p75 in regulating survival of some neural cell lines.

Recent studies allowed, neverthless, to conclude that the apoptotic actions of p75 is ligand-dependent (Majdan, et al., 1997), that signals apoptosis occur in a Trk-independent manner (Bredesen, et al., 1998) (Vaillant, et al., 1999) and that Trk activation silences p75 apoptotic signaling (Aloyz, et al., 1998) (Yoon, et al., 1998), leaving other p75 pathways 'intact'. In particular p75 mediates apoptosis signaling during neural injury and during development (Yoon, et al., 1998). Cell death signaling involved are JNK-p53-Bax (Vaillant, et al., 1999), NRIF (neurotrophin receptor-interacting factor) (Casademunt, et al., 1999), TRAF (tumor necrosis factor receptor-associated factor) (Khursigara, et al., 1999) and two novel finger motif proteins SC-1 (Schwann cell factor-1) (Chittka, et al., 1999) and NRAGE (neurotrophin receptor-interacting MAGE [melanoma antigen gene] homologue) (Salehi, et al., 2000) (Kaplan, et al., 2000).

3.4 Distribution and expression of neurotrophins and their receptors -

Through the differential expression and cellular localization of their receptors, neurotrophins can elicit diverse cellular functions in different types of neurons and at different cellular loci (Hyungju, et al., 2013). Indeed in contrast to other signaling system, the neurotrophins are not ubiquitously expressed. In addition, the neurotrophins are primarly localized in the target areas innervated by the responsive peripheral nervous system (PNS) axons (Davies, 1994). BDNF, NT-3 and NT-4/5 and their receptors, TrkB and TrkC, are widely and specifically distributed in the CNS, whereas NGF and its receptor TrkA expression is restricted to defined areas of the CNS such as striatal and basal forebrain cholinergic neurons. BDNF and NT-3 and their cognate receptors, TrkB and TrkC, are particularly highly expressed in the cerebellum, hippocampus and cerebral cortex, all well-studied sites of both developmental and adult synaptic plasticity

(McAllister, 1999). TrkC is also typically expressed early in development (Tessarollo, et al., 1993). In situ hybridization studies further indicate that full-length, kinase-containing forms of TrkB and TrkC are expressed, and often co-expressed, on most CNS neurons but not on non-neuronal cells. In contrast, truncated, non-catalytic forms of both TrkB and TrkC are found in both neurons and glial cells (McAllister, 1999). P75 is co-expressed with the Trk receptors in many neuronal populations (Chao, et al., 1995). During postnatal development, p75 is downregulated in most parts of the CNS but is rapidly induced after nerve lesion or seizure (Roux, et al., 1999). Finally, p75 is also expressed by different types of cells by the time they become postmitotic cells and/or migrate (Farinas, et al., 1998).

Neurotrophins and their receptors are not only expressed in neuronal tissues but also in non-neuronal tissues and various cell types such as developing heart (Clegg, et al., 1989), spleen (Yamamoto, et al., 1996), atherosclerotic vessels (Donovan, et al., 1995), macrophages (Barouch, et al., 2001), lymphocytes (Kerschensteiner, et al., 1999), endothelial cells (Nakahashi, et al., 2000) and vascular smooth muscle cells (Donovan, et al., 1995). For example, TrkA receptor is expressed by monocytes and its expression decreases during their differentiation into macrophages (Ehrhard, et al., 1993). TrkB receptor, instead, is expressed in muscle, heart and the vasculature (Scarisbrick, et al., 1993) (Timmusk, et al., 1993) (Donovan, et al., 1995) (Hiltunen, et al., 1996). In pathological states, TrkB expression is induced in neointimal vascular smooth muscle cells of the adult rodent and human aorta following vascular injury (Donovan, et al., 1995), while its haploinsufficiency is associated with a decrease in ApoE-null mice, in lesion development in the advanced stages of atherogenesis (Kraemer, et al., 2005). All together these data suggest that NTs have diverse roles even in non-neuronal tissues.

3.5 Role of neurotrophins in cardiovascular system – *Role of neurotrophins in cardiovascular development and adult cardiovascular system.*

NTs and their receptors are expressed during the developing heart and vessels (Bernd, et al., 2004) (Scarisbrick, et al., 1993). In particular, BDNF deficiency reduces endothelial cell-cell contact in the mouse embryonic heart, thus leading to intraventricular wall hemorrhage and depressed cardiac contractility (Donovan, et al., 2000). Similarly, $TrkB^{-/-}$ mice show a marked reduction of blood vessel density predominantly in the subepicardial region of

the developing heart (Wagner, et al., 2005). NT-3$^{-/-}$ mice develop abnormalities of the great vessels (Donovan, et al., 1996) (Tessarollo, 1998). Moreover, genetic deficiency of either NT-3 or TrkC impaired cardiac morphogenesis in mice (Donovan, et al., 1996) (Tessarollo, et al., 1997). Some of these developmental defects appeared as early as embryonic day (Arevalo, et al., 2006) (Anand, et al., 1996), which is before the onset of cardiac innervation in mice, thus suggesting the existence of a direct control of NTs on cardiovascular development (Tessarollo, 1998). Also p75^{NTR} is present in endothelial cells and vascular smooth muscle cells of large blood vessels during prenatal development (Von Schack, et al., 2001). Indeed, global null mice lacking all p75^{NTR} (p75^{NTR}ExonIV$^{-/-}$) show thinner wall and increased lumen diameter of the dorsal aorta; moreover, many p75^{NTR}ExonIV$^{-/-}$ embryos show vascular ruptures and cell leakage (Von Schack, et al., 2001).

It has been demonstrated that neurotrophins play key role also in adult cardiovascular system. Specifically NTs are expressed in endothelial cells (Kim, et al., 2004), where promote angiogenesis processes (Santos, et al., 1991) (Raychaudhuri, et al., 2001) (Cantarella, et al., 2002) (Nakamura, et al., 2006) (Kermani, et al., 2005) (Caporali, et al., 2009), in vascular smooth muscle cells (Scarisbrick, et al., 1993), where regulate chemotactic action (Donovan, et al., 1995), and in cardiac myocytes (Hohn, et al., 1990) (Maisonpierre, et al., 1990) (Selby, et al., 1987) (Timmusk, et al., 1993), where favor prosurvival signaling (Caporali, et al., 2008).

Role of neurotrophins in non-neuronal pathology. Considering the actions mediated by NTs, it has been demonstrated their link to some pathologies. Being strong promoters of angiogenesis and able to activate VEGF-A and metalloproteases, NTs contribute to the growth and diffusion of cancers (Bergers, et al., 2000) (Duffy, et al., 2008) (Jain, et al., 2007) (Nico, et al., 2008) and are associated with chronic inflammation (Raychaudhuri, et al., 2001) (Raychaudhuri, et al., 2004) (Rihl, et al., 2005). Notably, NTs are recruited also in inflammation after MI (Cai, et al., 2005), are expressed in vascular smooth muscle cells of human atherosclerotic plaque (Kraemer, 2002) where may contribute to plaque instability (Ejiri, et al., 2005) and finally, their expression is dramatically upregulated by arterial balloon injury (Donovan, et al., 1995).

3.5.1 Brain-derived neurotrophic factor (BDNF) and its cardiovascular functions

- BDNF has a complex gene structure, which has been documented and

revisited extensively (Timmusk, et al., 1993) (Aid, et al., 2007) (Liu, et al., 2005). Briefly, the human BDNF gene consists of several 5' exons with independent promoters. The 3' coding exon (exon IX) contains the sequence that codes for the pro-BDNF protein (*Figure 5*) (Boulle, et al., 2012).

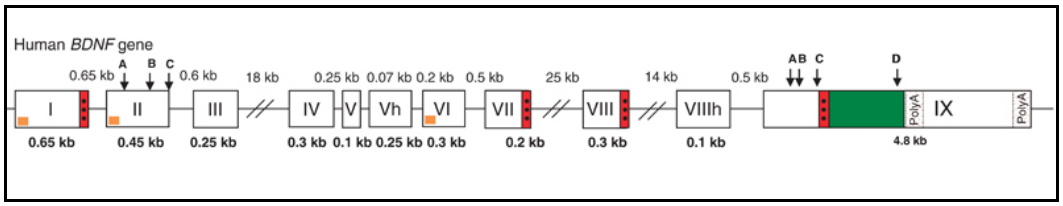

Figure 5 Human BDNF gene structure. Exons are represented as boxes and the introns as lines. Numbers of the exons are indicated in roman numerals and the size of exons and introns is indicated in arabic numerals. The 3´ coding exon (exon IX) contains two polyadenylation sites (poly A). The red box represent the start codon ATG that mark the initiation of transcription. The green box shows the region of exon IX coding for the pro-BDNF protein. Some exons, like exon II and IX, contain different transcript variants with alternative splice-donor sites (A, B, C, D).

BDNF is enriched in the central nervous system and is very important for the fetal development of the central nervous system, neuron plasticity and the preservation of memory in adulthood (DeFreitas, et al., 2001) (Chao, 2003). The expression or secretion of BDNF in the central nervous system is altered in neurodegenerative and psychiatric diseases (Pezet, et al., 2004) (Huang, et al., 2003) (Qian, et al., 1998) and consequently is considered a good target for the aetiology of MDD (Duman, et al., 1997) (Eikelis, et al., 2006). It has been demonstrated that BDNF is also produced in non-neuronal tissues. Low levels of BDNF mRNA and BDNF are detected in the thymus, liver, spleen, heart and lung (Ernfors, et al., 1990) (Katoh-Semba, et al., 1997) (Maisonpierre, et al., 1990) (Maisonpierre, et al., 1991). Lymphocytes and monocytes produce BDNF (Edling, et al., 2004) (Kerschensteiner, et al., 1999) (Schulte-Herbruggen, et al., 2005) and eosinophils utilize self-produced BDNF via the autocrine system to survive, evoking and extending the allergic reaction (Nockher, et al., 2005) (Raap, et al., 2005). Furthermore, BDNF also contributes to tumor cell growth and functions as an angiogenesis factor in circulating blood (Pearse, et al., 2005) (Yang, et al., 2006). The heterogenous distribution of BDNF, suggest its other functions, often not strictly related to neurological area.

Firstly, platelets contain abundant BDNF at a level comparable to that of the serum (Fujimura, et al., 2006) (Yamamoto, et al., 1990). The serum BDNF level is approximately ten

times higher than that of plasma because platelets release BDNF during the clotting process (Radka, et al., 1996) (Fujimura, et al., 2006) (Lommatzsch, et al., 2005) (Karege F, 2005a). From those findings, it is thought that platelets are important cells to store BDNF secreted from other tissues. Tamura et al. demonstrated that BDNF release from platelets occurs after stimulation with PAR1 activation, human platelets have two distinct pools of BDNF, in the α-granules (Lorgis, et al., 2009) (Pliego-Rivero, et al., 1997) and cytoplasm and BDNF contained in α-granules is that release after stimulation, not that of the cytoplasm (Tamura, et al., 2011). The origin of platelet BDNF is still elusive. It was suggested that BDNF is taken up into platelets through an uncharacterized transport mechanism (Fujimura, et al., 2006), or translated in platelets from mRNA derived from megakaryocytes (MK) (Tamura, et al., 2012) or taken up from the circulation. However, debate is the ability of megakaryocytes to express and/or produce BDNF. In fact, MEG-01, a megakaryocytic cell line, neither express BDNF and lack the ability to produce BDNF under autonomous proliferating conditions in absence of cytokines (Fujimura, et al., 2006); in contrast, MEG-01 stimulated/differentiated with thrombopoietin (TPO) show a great ability to produce BDNF (Tamura, et al., 2012). However, since the release of BDNF from platelets is strictly correlated to the release of VEGF, it has been supposed a pro-angiogenic function of BDNF, as confirmed by other studies (Yang, et al., 2006) (Donovan, et al., 2000) (Kermani, et al., 2005) (Kim, et al., 2004).

Secondly, BDNF can modify inflammation pattern; indeed the BDNF receptor, TrkB, is increased in the heart of aging rodent; moreover functional studies revealed that age-associated alterations in cardiac BDNF-mediated pathways could enhance inflammation and increase MI in the aging heart (Cai, et al., 2005). Moreover, increased inflammation is associated to a greater infiltration of mononuclear cells. Remarkable, the intramyocardial administration of BDNF induces an increase in inflammation and macrophages activity (Lorgis, et al., 2009). Further, BDNF favours macrophage phagocytosis and promotes the release of some inflammatory cytokines, such as interleukin (IL)-1β (Asami, et al., 2006). Moreover, modifications in the expression and production of BDNF have been detected in both chronic inflammatory diseases and in atherosclerosis (Chaldakov, et al., 2001) (Kerschensteiner, et al., 1999). In particular, it has been shown that BDNF and its receptor TrkB are strongly expressed in atherosclerotic vessels, in ischemic myocardial tissue and in ischemic limbs tissue of human and/or

animal models. These increases are associated with mononuclear cell infiltration (Kermani, et al., 2007) and activation of matrix-degrading proteases MMP-2 and MMP-3, and stimulation of uPA and PAI-1 production by endothelial cells (Pepper, 2001) (Sun, et al., 2006). BDNF/TrkB activation pathway seems to be involved in the formation of atherosclerotic plaque and in the revascularization of ischemic tissues, playing a complex role in the cardiovascular dysfunction and in angiogenic processes. Indeed, Kraemer et al. (Kraemer, et al., 2005) show that the decreased expression of neurothrophin TrkB receptor reduces lesion size in the apolipoprotein E-null mutant mouse, whereas Kermani et al. (Kermani, et al., 2007), in a model of peripheral ischemia, show that BDNF enhances capillary formation increasing local TrkB-expressing endothelial cells and the recruitment of subpopulation of myeloid cells, which may contribute to vessel formation or stabilization.

BDNF has been implicated in glucose and lipid metabolism, energy homeostasis and in metabolic syndrome (Geroldi, et al., 2006) (Han, et al., 2008) (Saito, et al., 2009). Actually patients heterozygous for BDNF deletions showed higher Body Mass Index (BMI) z score throughout childhood and by 10 years of age, 100% of the patients are obese (Han, et al., 2008). Similarly, individuals with obesity and type 2 diabetes have low circulating levels of BDNF (Krabbe, et al., 2007), as well as there is an inverse association between the peripheral BDNF concentration and BMI in children and adults (Lommatzsch, et al., 2005). Studies carried out on murine models have supported all these observations. In fact, both heterozygous for targeted disruption of BDNF ($BDNF^{+/-}$ mice) (Kernie, et al., 2000), and the reduction in the expression of TrkB (in partial KO mice) (Xu, et al., 2003) lead to obesity in mice as consequences of increased food intake. Intracerebroventricular (ICV) infusion of BDNF into $BDNF^{+/-}$ mice normalizes food intake, body weight and activity, suggesting a physiological role of BDNF in regulating food intake (Rothman, et al., 2012).

Considerable amount of research effort has been devoted to understand the role of BDNF in the pathogenesis and/or recovery of major types of cardiac diseases. BDNF is essential for the development of the autonomic nervous system (ANS), in particular in the formation of synaptic connectivity with peripheral target and for the survival of arterial baroreceptors during vascular innervation (Brady, et al., 1999). Recent findings indicate that BDNF plays a major role in ANS control of cardiovascular function in adults. Increased levels of BDNF in CNS (Lee, et al., 2002) (Neeper, et al., 1996), induced by

both exercise and dietary energy restriction, modulate ANS with consequent decreased heart rate and increased heart rate variability (Rosenwinkel, et al., 2001) (Wan, et al., 2003b). Finally, $BDNF^{+/-}$ mice, which exhibit a 50% in BDNF mRNA, have significantly elevated heart rates compared to WT mice. Further, when exposed to restraint stress, $BDNF^{+/-}$ mice fail to elevate their heart rate to WT levels, indicating an impaired cardiovascular stress response (Rothman, et al., 2012).

However, while some studies have demonstrated a reduction of NGF expression in human atherosclerotic plaques (Chaldakov, et al., 2001), other have shown marked expression of BDNF in the atheromatous intima and adventitia, macrophages and smooth muscle cells in atherosclerotic coronary arteries in people with unstable angina (Ejiri, et al., 2005). In particular, the stimulation with recombinant BDNF significantly enhanced oxidative stress in cultured human coronary artery smooth muscle cells, suggesting that BDNF is involved in the mechanism of oxidant injury and supporting the observation that increased coronary levels of BDNF was linked to platelet activation and inflammatory response (Lorgis, et al., 2009).

In addition, circulating levels of BDNF are proposed as predictor risk factor for CVD. Plasma BDNF level is reported to decrease in subjects with acute coronary syndrome (Manni, et al., 2005). In contrast, significantly greater serum BDNF levels are found in the coronary sinus blood samples in subjects with unstable angina compared to those with stable angina or healthy controls and in patients with myocardial infarction, and these levels correlated positively with soluble P-selectin, an index of platelets activation (Ejiri, et al., 2005) (Lorgis, et al., 2009). Recently, plasma BDNF levels have been inversely associated with the levels of triglyceride, LDL-cholesterol and fibrinogen, male sex and age and positively with high-density lipoprotein cholesterol level and platelet count in people with angina pectoris. Interestingly, plasma BDNF level was as an independent predictor of 4-year coronary and all-cause mortality, indicating that low plasma BDNF may be associated with future coronary events and death in these patients (Jiang, et al., 2011).

At the light of these data, it is possible conclude that BDNF regulates many physiological and pathological non-neuronal events.

3.5.1.1 BDNF Val66Met polymorphism - The recent discovery of a single-nucleotide polymorphism (SNP) in the *bdnf* gene (Val66Met), found only in humans, leading to a methionine (Met) substitution for a valine (Val) at codon 66 in the pro-domain (*Figure 6*), has provided a valuable tool to assess potential contribution of BDNF to affective disorders.

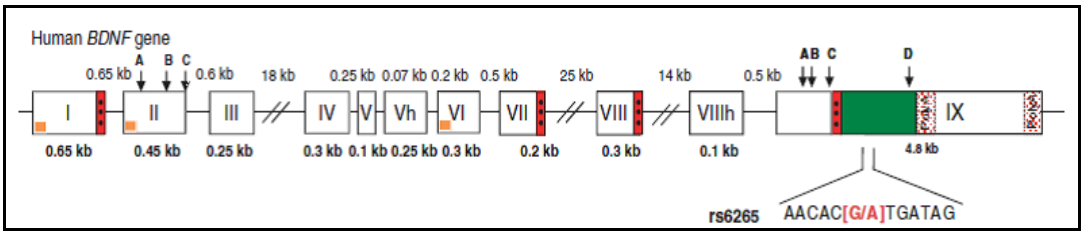

Figure 6 Structure of the human BDNF gene. The green box shows the region of exon IX coding for pro-BDNF gene protein, including the rs6265 genetic variant implicated in the Val66Met polymorphism.

This polymorphism is common in human population with an allele frequency of 20 to 30% in Caucasian population (Schimizu, et al., 2004). Human heterozygous for the Met allele have smaller hippocampal volumes (Bueller, et al., 2006) (Pezawas, et al., 2004) (Szeszko, et al., 2005) and perform poorly on hippocampal-dependent memory tasks (Hariri, et al., 2003) (Egan, et al., 2003). The mechanisms that contribute to alter $BDNF_{Met}$ function have been studied in neuronal culture systems. The distribution of $BDNF_{Met}$ to neuronal dendrites and its activity-dependent secretion are decreased (Egan, et al., 2003) (Chen, et al., 2005) (Chen, et al., 2004). These trafficking abnormalities are likely to reflect impaired binding of $BDNF_{Met}$ to a sortilin, a sorting protein, that interacts with BDNF in the prodomain region that encompasses the Met substitution (Chen, et al., 2005). This polymorphism has been associated to an altered susceptibility to various neuropsychiatric diseases, including Alzheimer's disease (Ventriglia, et al., 2002) Parkinson disease (Momose, et al., 2002), schizophrenia (Neves-Pereira, et al., 2005) (Rosa, et al., 2006), schizoaffective disorder (Lencz, et al., 2009) and bipolar disorder (Sklar, et al., 2002) (Muller DJ, 2006). The effect of the polymorphism on the circulating blood of BDNF is still unclear and controversial. Some studies did not find any difference among plasma levels in Met/Met and Val/Val or Val/Met subjects (Tramontina, et al., 2007) (Karege F, 2005a) (Terracciano, et al., 2010); while other groups demonstrated that the polymorphism is associated to increased levels of serum BDNF (Lang, et al., 2009). However, some studies have demonstrated patients affected by major depression show decreased serum BDNF

levels and more specifically a down-regulation of mature form of the neurotrophin (mBDNF), while plasma BDNF levels are often unchanged (Bocchio-Chiavetto, et al., 2010), indicating an altered release of BDNF.

Some studies have demonstrated the existence of the link between the polymorphism and cardiovascular diseases. A recent finding has demonstrated that BDNF Val66Met polymorphism is associated with unstable angina pectoris (UAP) in the population of Han Chinese, and it seems that the $BDNF_{Met/Met}$ genotype has a protective effect on the occurrence of UAP (Jiang, et al., 2009). On the other hand, Bozzini et al. (Bozzini, et al., 2009) suggest an involvement of the Met/Met genotype in the pathogenesis of CAD, associated with depression, in women, arguing a protective role of Val/Val genotype against CAD and against CAD associated with depression.

Finally, Alexander and collegues highlighted a significant effect of the BDNF Val66Met polymorphism on hypothalamic-pituitary-adrenal (HPA)-axis reactivity to a standardized laboratory stress task in healthy male adults, pointing to an attenuated cortisol response in met-allele carriers when compared to subjects homozygous for the val-allele. Moreover, the same picture emerges with regard to heart rate reactivity as an indicator for the sympathetic activation. Based on these findings, the authors tempt to speculate that reduced activity-dependent secretion of BDNF in met-allele carriers results in an attenuated BDNF release to endocrine and cardiovascular stressful situations (Alexander, et al., 2010).

In conclusion, in consideration of the heterogeneity of data available in literature, the impact of BDNF Val66Met polymorphism on CVD must still be extensively studied.

2 AIM OF THE STUDY

Evidence suggests a strong link between neurotrophin and cardiovascular disease (CVD) (Caporali, et al., 2009).

The brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors that promotes neuronal survival, differentiation and maintenance (Donovan, et al., 2000), is involved in the pathogenesis of different neurodegenerative diseases (Zuccato, et al., 2009). BDNF and its receptor, the tyrosine receptor kinase B (TrkB), are also expressed in non-neuronal cells, such as activated macrophages and lymphocytes (Nakahashi, et al., 2000), platelets (Fujimura, et al., 2006) (Karege F, 2005a), endothelial and vascular smooth muscle cells (Donovan, et al., 2000). BDNF plays a critical role in regulating both vascular development (Kermani, et al., 2007) and response to injury (Lewin, et al., 1996).

Indeed, it is enriched in atheromatous intima and around adventitial vasa vasorum, while hardly detectable in non-atherosclerotic arteries (Ejiri, et al., 2005). Intriguingly, reduced levels of BDNF have been associated with increased coronary events and mortality in patients affected by acute coronary syndrome (Manni, et al., 2005).

An amino acid substitution at codon 66 (BDNF Val66Met) that affects BDNF availability and function (Chen, et al., 2004), is related to stress vulnerability (Ventriglia, et al., 2002) (Momose, et al., 2002) (Sen, et al., 2003) (Neves-Pereira, et al., 2002) and recently it has been suggested to be linked to CVD (Bozzini, et al., 2009) (Jiang, et al., 2009). No information is available on a role of BDNF in thrombosis. We hypothesize that a genetic variant of BDNF gene, underlie an increased susceptibility to thrombotic events. In particular we focused on analysing the different actors involved in the thrombotic events: the coagulation cascades, platelets and vessel wall.

To achieve this goal, the experiments have been carried out in mice generated with a variant BDNF mouse ($BDNF^{Met/Met}$) that reproduces the phenotypic hallmarks in humans with the variant allele. $BDNF^{Met/Met}$ mice, considered a good model of depression, when placed in conflict settings (Chen, et al., 2004), display increased anxiety-related behaviours, caused by a reduction of its trafficking; thus representing not only a good model to understand the role of neurotrophins in thrombosis but also to clarify the emerging link among depressive disorders and cardiovascular disease.

3 RESULTS

1 Effect of BDNF Val66Met mutation on arterial thrombosis and pulmonary thromboembolism

The physiological relevance of BDNF Val66Met mutation on thrombosis was studied in BDNF Val/Val (wild type) and BDNF Met/Met mice, both genders and age of 10-12 weeks. In particular, we used two different thrombosis models: ***a***) the carotid artery $FeCl_3$ (10%) injury model and ***b***) the thromboembolism model. In the carotid artery injury model the thrombus formation was evaluated as flow variation, monitored during a 30 minutes-period after the application of the stimulus. It was considered a stable occlusion when we observed a decrease in flow of ≥90% from baseline for ≥5 minutes, during which time the flow did not change by > 1% of baseline per second. No significant difference in terms of basal levels of blood flow was noted between mouse genotypes (**Val/Val**: 0.97 ± 0.01 and **Met/Met**: 0.96 ± 0.04 ml/min; p=0.42). Topical application of $FeCl_3$ induced a stable clot occlusion in 83.3% of Met/Met mice. In contrast, all Val/Val mice developed only a partially carotid artery occlusion (48%) within the same 30-minutes observation period. In particular, in Met/Met mice, blood flow was dramatically reduced compared with Val/Val mice within 10 minutes by the application of $FeCl_3$ with a markedly shorter carotid occlusion time (*Figure 1A and B*), indicating an increased propensity to thrombosis in these animals compared to Val/Val mice. The strong differences observed in terms of carotid arterial thrombus formation between Val/Val and Met/Met mice were further confirmed by histological analysis (*Figure 1C*).

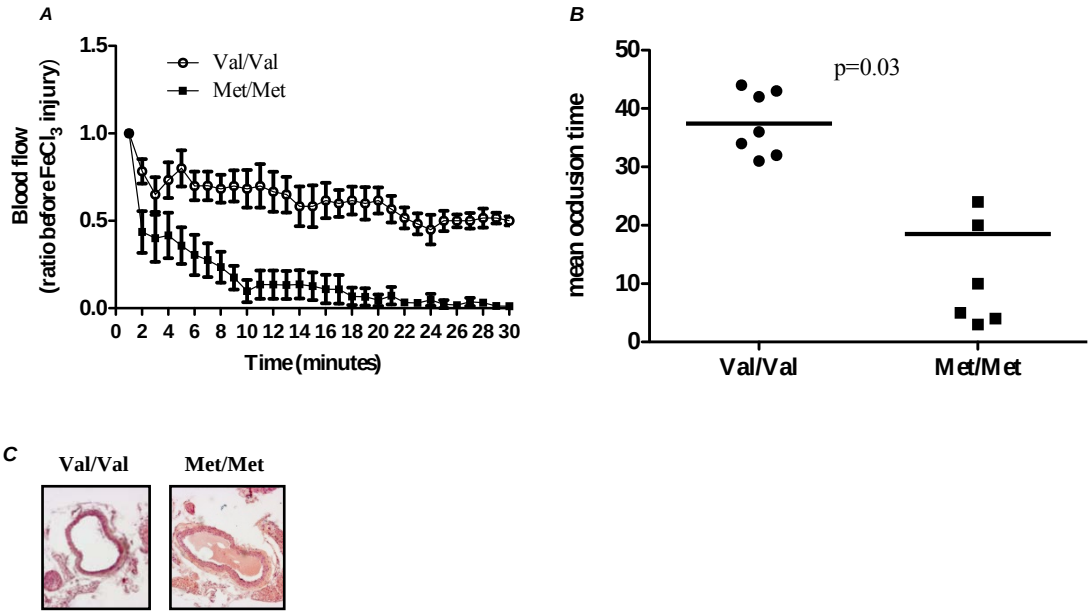

Figure 1 Ferric chloride-induced thrombosis. ***A***) Blood flow was monitored for a period of 30 minutes after application of ferric chloride on the carotid artery of Val/Val and Met/Met mice; ***B***) mean occlusion time of Val/Val (open circles) and Met/Met (black squares) mice; ***C***) histological analysis of thrombi isolated from Val/Val and Met/Met mice (n=6-7 mice per group). Data shown are mean ± SEM.

The increased thrombotic phenotype of Met/Met mice was also confirmed in a pulmonary thromboembolism model, in which platelet-rich clots are triggered by rapid intravenous administration of a mixture of collagen and epinephrine. Mice were observed for 15 minutes and the time of cessation of breathing stasis recorded. The percentage of mortality in Met/Met mice, within 15 minutes after collagen/epinephrine injection, was significantly higher compared with their Val/Val littermates (**Val/Val**: 2.0 % ± 0.02 and **Met/Met**: 30.0 % ± 0.1; $p < 0.05$) (*Figure 2*).

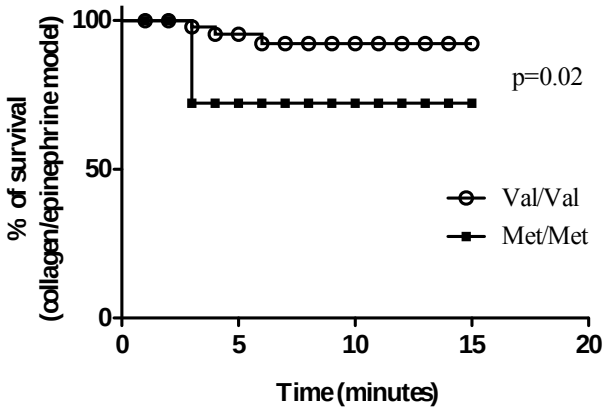

Figure 2 Thromboembolism model. Percentage of survival of Val/Val and Met/Met mice after infusion of a collagen and epinephrine mixture (p= 0.02; n=6 mice per group). Data shown are mean ± SEM.

These results indicate that BDNF Val66Met polymorphism predisposes to thrombotic events *in vivo*.

2 Blood cell counts and platelets parameters in Val/Val and Met/Met mice

Blood cell counts from Val/Val and Met/Met mice were performed. Similar number of red blood cells was counted in the two groups of animals (*Table 1*). In contrast, very higher number of platelets (*Table 1*) was measured in Met/Met mice. Moreover, Met/Met mouse platelets have similar size (*Table 1*), but a significant, even if mild, higher percentage of reticulated platelets compared to Val/Val mice (*Table 1*).

In addition, Met/Met mice showed a greater number of circulating leukocytes compared to Val/Val mice (*Table 1*). Considering the heterogeneity of leukocyte populations and their different biological roles, we performed counts of neutrophil granulocytes, monocytes and lymphocytes on whole blood of Val/Val and Met/Met mice. We observed that neutrophil (*Table 1*) and monocyte (*Table 1*) counts were significantly higher in Met/Met mice than in control mice, while lymphocytes were similar in the two groups of animals (*Table 1*).

	Val/Val	Met/Met
Erythrocytes (10^6/μl)	15.3 ± 0.8	15.3 ± 0.8
Leukocytes (#/μl)	2523.0 ± 140.1	3810.0 ± 253.4***
Neutrophil granulocytes (#/μl)	408.3 ± 47.4	706.5 ± 109.6*
Monocytes (#/μl)	48.3 ± 3.1	184.0 ± 34.0*
Lymphocytes (#/μl)	2168.0 ± 154.4	2998.0 ± 473.6
Platelets N° (10^3/μl)	1046.0 ± 38.5	1296.0 ± 53.5***
MPV fL	6.5 ± 0.06	6.5 ± 0.2
% reticulated platelets	13.5 ± 0.6	15.4 ± 0.8*

Table 1 Effect of BDNF Val66Met polymorphism on blood cell counts and platelets parameters. MPV: mean platelet volume (n=6-12 mice per group). Data shown are ± SEM. *p<0.05; **p<0.01; ***p<0.001.

The high platelets number associated with increased leukocytes number suggest a pro-thrombotic and pro-inflammatory phenotype of Met/Met mice.

3 Evaluation of hemostatic system in Val/Val and Met/Met mice

To evaluate a possible alteration in the hemostatic system of Met/Met mice, rotation thromboelastography (ROTEM®) analysis were performed. This method assesses the combined influence of circulating plasmatic and cellular (platelets, red blood cells, leukocytes) elements on clot formation, including platelet function and fibrin formation in whole blood, evaluating the viscoelasticity of clot during all phases of its formation.
In particular, we considered only some parameters obtained from the thromboelastographic trace, i.e. coagulation time (CT), clotting formation time (CFT), maximum clot firmness (MCF) and maximum clot elasticity (MCE).
In addition, we carried out specific tests to evaluate the activity of coagulation factors and of Tissue Factor (TF), the functionality of platelets and fibrinogen.

Recalcification test (NATEM), induced by the addition of high concentration of $CaCl_2$, showed that CFT was significantly shorter in Met/Met mice and that MCF and MCE in Met/Met mice increased nearly 3 times compared to Val/Val mice (*Table 2*). In contrast,

CT was almost the same between the two different groups of mice. In conclusion, NATEM test provides evidence that whole blood clot stability and elasticity is increased in Met/Met mice compared to Val/Val mice and it suggests an alteration in platelets and fibrinogen activity in homozygote transgenic Met/Met mice.

Two specific thromboelastographic tests to evaluate the contribution of ***extrinsic (EXTEM) and intrinsic (INTEM) pathways of the coagulation*** were performed. From these tests emerged that: CT was similar in the two groups of animals analyzed in both tests, suggesting unchanged coagulation factors functionality. In contrast, CFT was shorter in Met/Met mice only in the EXTEM test, indicating altered platelets and/or fibrinogen functionality specifically linked to the activation of extrinsic pathway. However, statistical differences in terms of MCF and MCE were detected both in EXTEM and INTEM tests. In fact, blood from Met/Met mice showed a greater MCF and MCE than Val/Val mice (*Table 2*).
These results show that Met/Met mice form greater and more stable clot, due probably to higher functionality of platelets and fibrinogen.

	NATEM		**INTEM**		**EXTEM**	
	Val/Val	Met/Met	Val/Val	Met/Met	Val/Val	Met/Met
CT	321.4 ± 22.4	287.8 ± 23.1	148.9 ± 7.2	154.9 ± 5.9	31.8 ± 2.5	27.7 ± 1.2
CFT	146.1 ± 13.6	105.3 ± 12.3*	43.4 ± 2.3	44.5 ± 3.3	72.8 ± 1.7	62.1 ± 0.8**
MCF	55.9 ± 1.9	62.0 ± 0.8*	64.9 ± 1.1	68.0 ± 0.5*	60.7 ± 1.0	64.1 ± 1.9**
MCE	130.1 ± 10.5	164.1 ± 5.6*	187.6 ± 8.1	212.9 ± 4.3*	156.0 ± 7.1	181.5 ± 6.8**

Table 2 Thromboelastographic analysis. Test NATEM, INTEM and EXTEM on whole blood of Val/Val mice and Met/Met mice (n=10 mice per group). Data shown are ± SEM. * $p<0.05$, ** $p< 0.01$.

3.1 Coagulation factors activity – To verify the results suggested by thromboelastographic analysis, related to normal activity of coagulation factors in Met/Met mice (similar CT in each test performed), we carried out a pro-coagulant activity test (PCA) in presence of factor deficient plasma to confirm this hypothesis.
As expected, no alteration in the levels of each coagulation factor analyzed was observed (*Figure 3*).

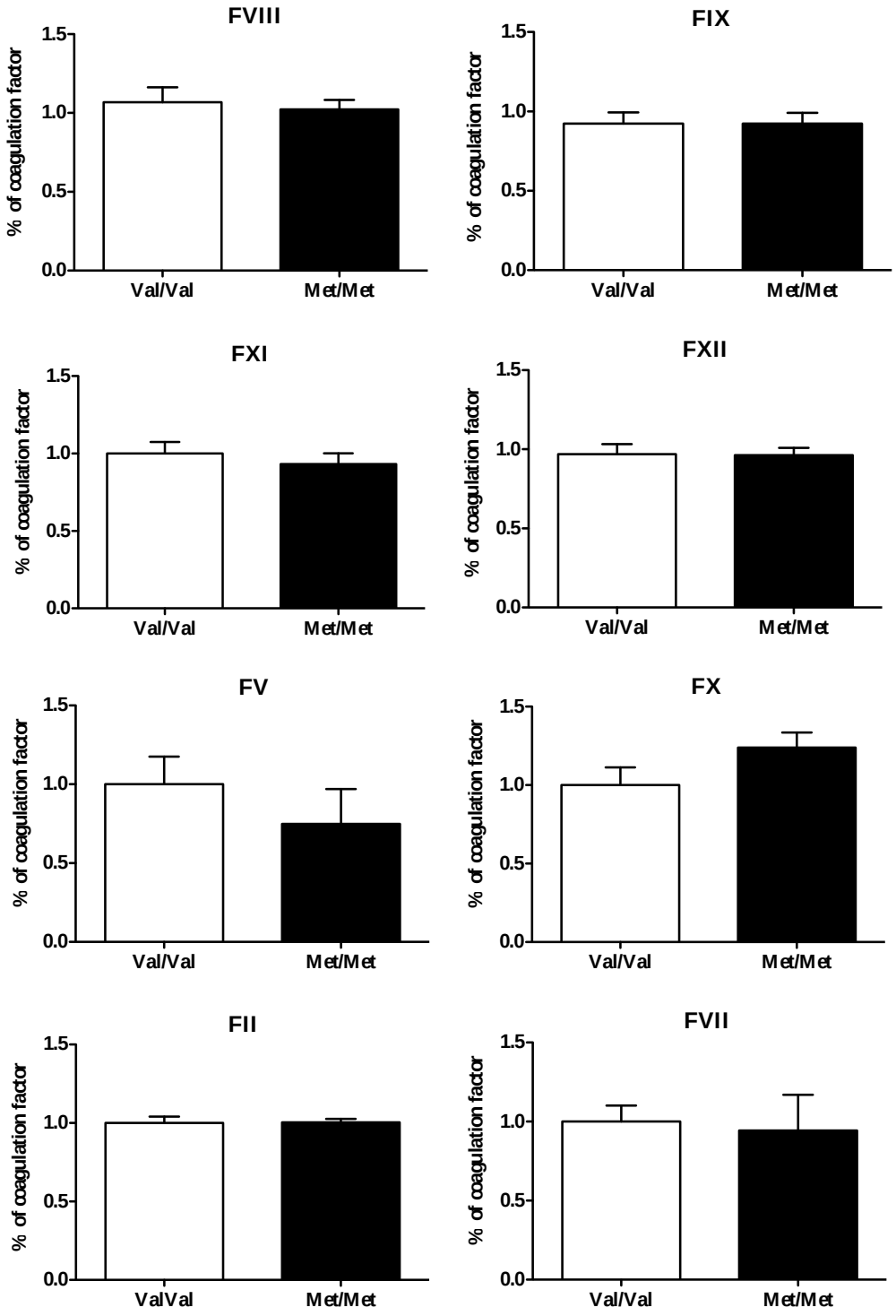

Figure 3 Dosage of coagulation factors of intrinsic (FVIII, FIX, FXI, FXII), common (FV, FX, FII) and extrinsic (FVII) pathways of coagulation by PCA test in Val/Val (open bars) and Met/Met (black bars) mice (n=10-11 mice per group). Data shown are ± SEM.

In addition, we measured TF activity, main activator of coagulation cascade on thrombosis, in plasma microparticles, and in circulating leukocytes. The levels of microparticles TF activity were similar in Met/Met and Val/Val mice isolated from whole blood of Val/Val and Met/met mice, revealing only a trend of increasing in Met/Met mice than Val/Val (*Figure 4A*). Moreover, we observed that leukocytes, main carriers of circulating TF (Cermak, et al., 1993), isolated from Met/Met mice, coagulated faster than those isolated from Val/Val mice, meaning higher TF units (U TF/μg protein) in leukocytes of Met/Met than in Val/Val mice (*Figure 4B*).

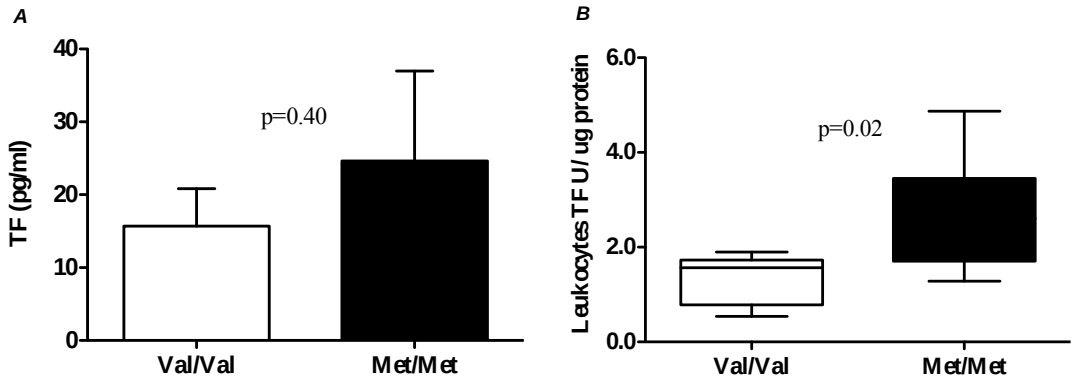

Figure 4 Dosage of Tissue Factor (TF) activity. ***A***) microparticles TF activity (n=10 mice per group) and ***B***) leukocytes TF activity (n=6-8 mice per group) in Val/Val (open bars) and Met/Met (black bars) mice. Data shown are ± SEM.

3.2 Platelets characterization – Since the shorter CFT obtained by thromboelastography in Met/Met mice associated with higher plasma levels of thrombospondin-1 (TSP-1), a protein released from α-granules of platelets (Jurk, et al., 2003), and increased serum thromboxane B2 (TXB_2) levels in Met/Met mice than Val/Val mice (*Figure 5A and B*), suggest a hyperactive platelet phenotype, we next characterized the platelets activation by different approaches.

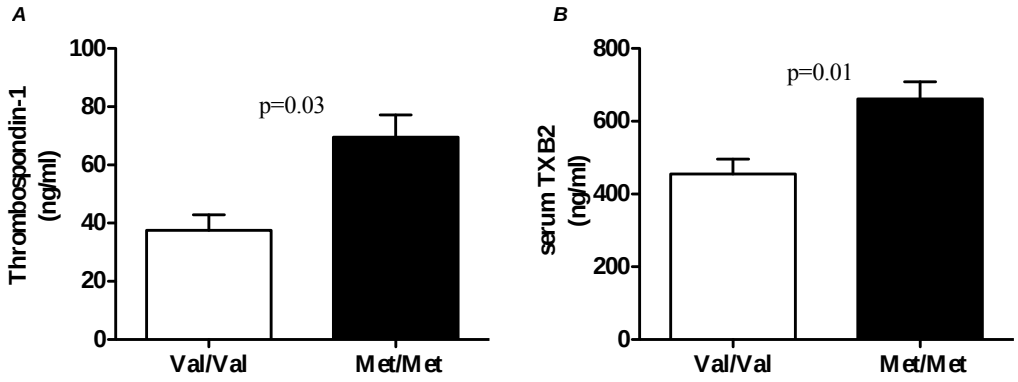

Figure 5 Dosage of prothrombotic mediators. ***A***) Plasma levels of Thrombospondin-1 and ***B***) Thromboxane B2 serum release in Val/Val (open bars) and Met/Met (black bars) mice. (n=6 mice per group). Data shown are mean ± SEM.

We firstly carried out the study of platelets aggregation in platelet rich plasma (PRP) obtained from Met/Met and Val/Val mice. A statistically significant difference in the threshold concentrations of collagen (0.0175 to 0.5 μg/ml) and thrombin (from 0.0125 to 0.05 U/ml) required to induce platelet aggregation was observed between the two groups of mice (*Figure 6A, B and C*).

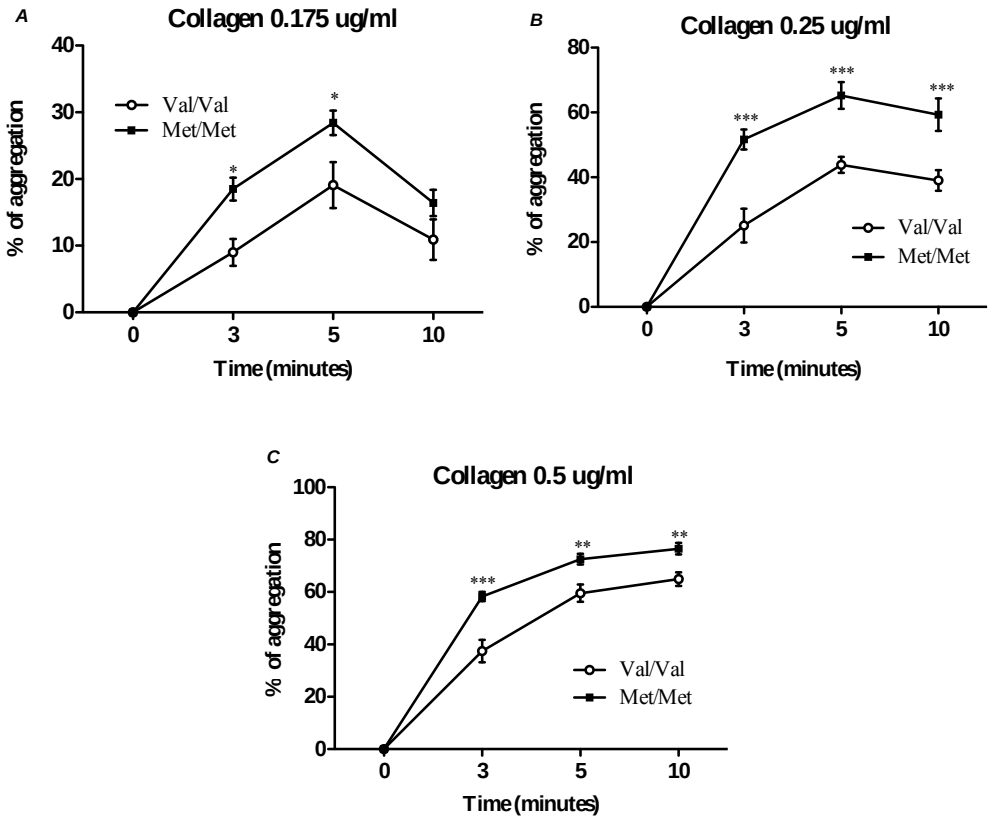

Figure 6 Effect of collagen on platelets aggregation. Platelets aggregation induced by ***A***) collagen 0.175 μg/ml, ***B***) collagen 0.25 μg/ml and ***C***) collagen 0.5 μg/ml in Val/Val (open circles) and Met/Met (black squares) mice (n=6 mice per group). Data shown are mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

In particular, at the lowest concentration of collagen we observed only a modest difference between the two groups, but collagen 0.25 μg/ml induced a strong Met/Met platelets aggregation (about 52%) already after 3 minutes of stimulation and reached a plateau (65%) at 5 minutes, whereas the maximal aggregation achieved by Val/Val platelets was only of 25% and of 44% at 3 and 5 minutes, respectively. In contrast, there were no differences in the aggregation induced by the highest concentration of collagen. Comparable results were obtained after activation of platelets with thrombin. Indeed if at the lowest concentration of thrombin we could not appreciate a relevant difference, after adding thrombin 0.025 U/ml Met/Met platelets reached an aggregation of 59% after 5 minutes while Val/Val platelets aggregated for only 41% (*Figure 7A , B and C*) after the same time.

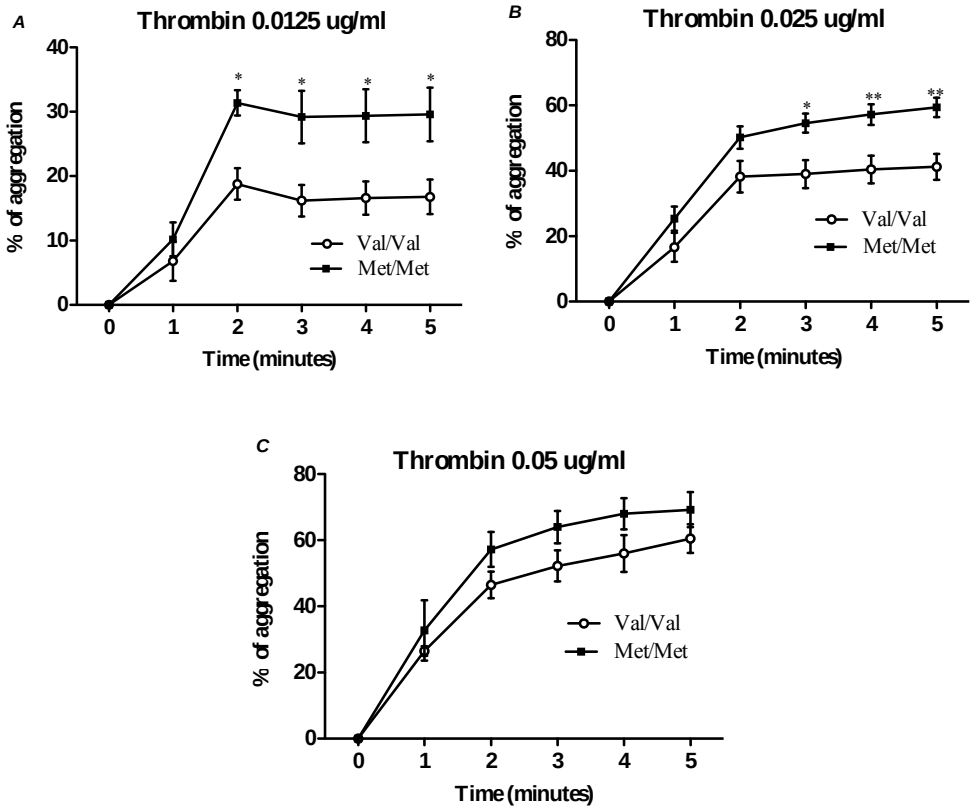

Figure 7 Effect of thrombin on platelets aggregation. Platelets aggregation induced by ***A***) thrombin 0.0125 U/ml, ***B***) thrombin 0.025 U/ml and ***C***) thrombin 0.05 U/ml in Val/Val (open circles) and Met/Met (black squares) mice (n=5 mice per group). Data shown are mean ± SEM. *p<0.05; **p<0.01

To better characterize the hyperactivation of platelets observed in Met/Met mice, we evaluated by flow cytometry the expression of P-selectin, receptor involved in the interaction of platelets with leukocytes (Hu, et al., 2010), and of GPIIbIIIa, receptor designated to the binding of fibrinogen (Savage, et al., 1996), on washed platelets isolated from Met/Met and Val/Val mice in response to two concentrations of thrombin (0.01 and 0.5 U/ml). P-selectin expression was higher in Met/Met mice than in Val/Val after the treatment with low concentration of thrombin (*Figure 8A*). The difference was lost when platelets were stimulated with thrombin 0.5 U/ml. On the other hand, receptor GPIIbIIIa expression was significantly increased in Met/Met mice for both thrombin concentrations used (*Figure 8B*).

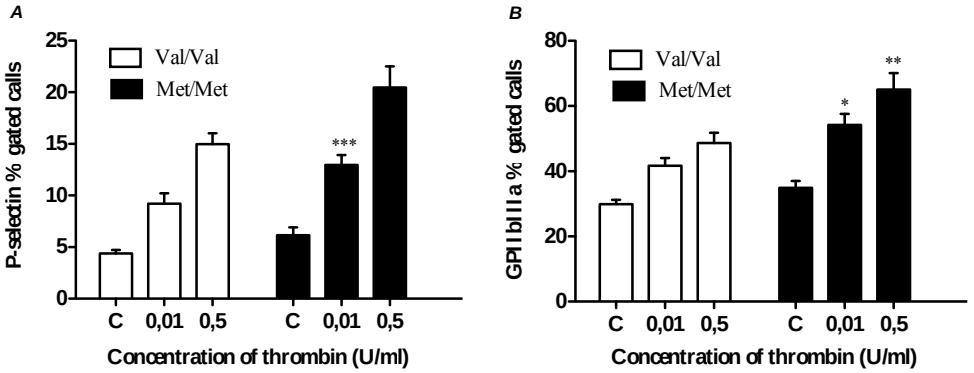

Figure 8 Flow cytometry analysis: platelets receptors. ***A***) P-selectin and ***B***) GPIIbIIIa receptors expression induced by thrombin on Val/Val (open bars) and Met/Met (black bars) platelets (n=10 mice per group). Data shown are mean ± SEM. *p<0.05; ***p<0.001.

The higher reactivity of Met/Met platelets was also indicated by the increased ability of platelets of binding fibrinogen after stimulation with Adenosine DiPhosphate (ADP 1 μM) (*Figure 9*).

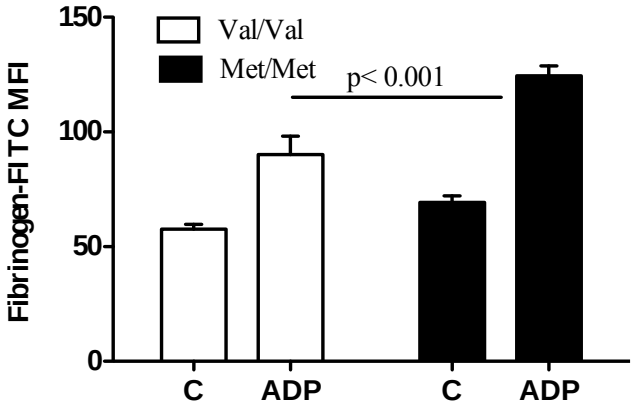

Figure 9 Flow cytometry analysis: fibrinogen binding. Ability of Val/Val (open bars) and Met/Met (black bars) platelets to bind fibrinogen at baseline and after treatment with ADP (n=7-8 mice per group). Data shown are mean ± SEM.

To determine the possible role of BDNF Val66Met polymorphism in outside-in signaling, we performed clot retraction assay, on PRP isolated from whole blood of Met/Met and Val/Val mice. Analysis of clot retraction kinetics showed a significant

difference between the two groups of animals, with a 35% reduction in the percentage of extruded serum, suggesting a defect in cytoskeleton dynamics (Egot, et al., 2013) (*Figure 10 Ai and Aii*).

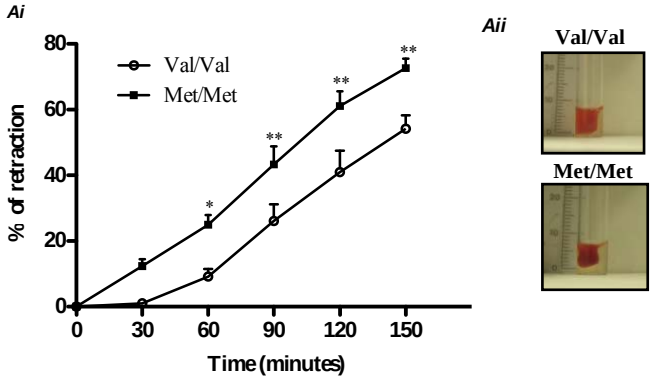

Figure 10 Clot retraction. ***Ai***) PRP isolated from Val/Val (open circle) and Met/Met (black bars) mice was coagulated by the adding of thrombin and the time of retraction was measured, ***Aii***) photographical representation of Val/Val and Met/Met clot retraction at 150 minutes (n=6 mice per group). Data shown are mean ± SEM. *p< 0.05; **p< 0.01.

Key processes, induced by fibrinogen engagement, are cell spreading and activation of retractile signaling pathways. We therefore allowed platelets from Met/Met and Val/Val mice to adhere on fibrinogen coated-coverlips from 10 to 40 minutes under basal conditions. The number of adherent platelets on fibrinogen coated-surfaces was significantly increased in Met/Met mice (*Figure 11A and B*) compared to Val/Val mice. In particular, we observed that already after 20 minutes of incubation the number of platelets adhered on fibrinogen is 2 fold higher in Met/Met if compared to Val/Val mice and this difference is maintained almost unchanged for following 20 minutes of observation.

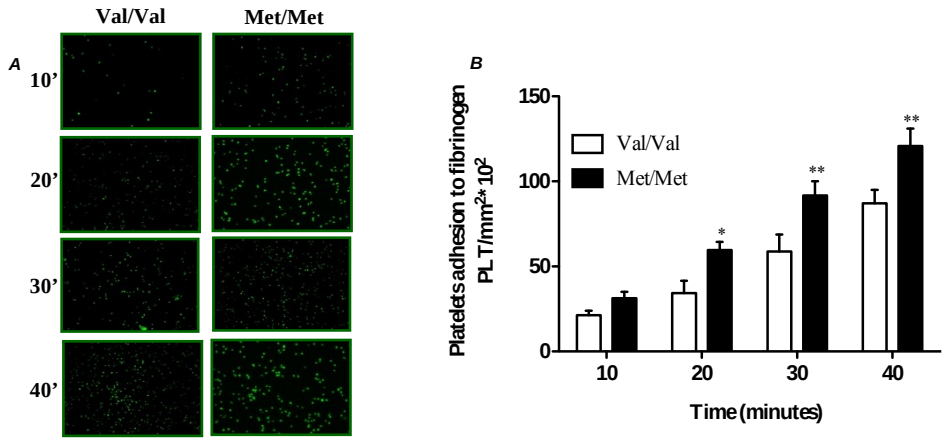

Figure 11 Platelets adhesion. Washed platelets of Val/Val (open bars) and Met/Met (black bars) mice let to adhere on fibrinogen coated-coverlips and then ***A***) observed and ***B***) counted by microscopy. Data shown are mean ± SEM of 6 independent experiments. *p< 0.05; ** p< 0.01.

Finally we evaluated by flow cytometry the percentage of platelet-leukocyte aggregates on whole blood of Met/Met and Val/Val mice under basal and ADP (5 μM) stimulated conditions. The percentage of platelet-leukocyte aggregates was significantly higher in Met/Met mice than controls in both conditions, in agreement with the data previously reported about P-selectin surface expression (*Figure 12Ai and Aii*).

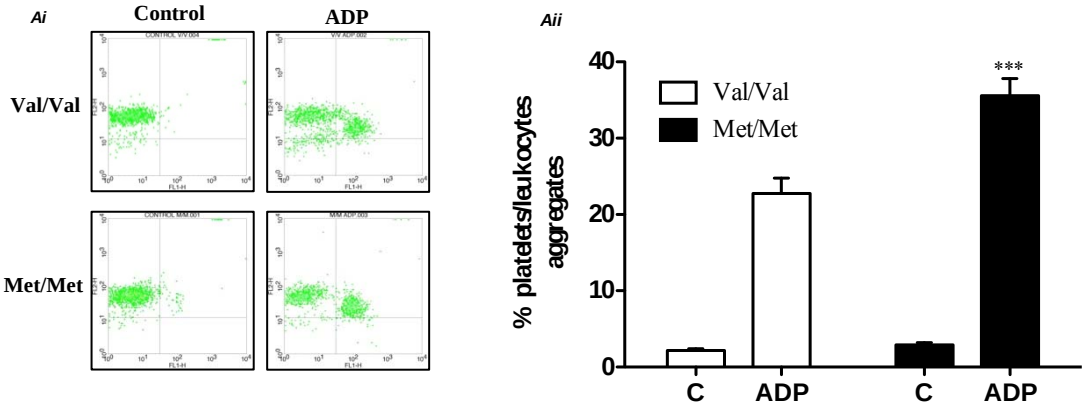

Figure 12 Flow cytometry analysis: number of platelet-leukocyte aggregates. ***Ai***) Graphical representation and ***Aii***) quantitation of platelet-leukocyte aggregates in Val/Val (open bars) and Met/Met (black bars) whole blood samples at baseline and after treatment with ADP; (n=8-9 mice per group). Data shown are mean ± SEM. *** p< 0.001.

3.3 Contribute of fibrinogen - To determine the contribution of fibrinogen in hemostatic system in our animal model, we used two different approaches: ***a***) FIBTEM

test performed in whole blood samples and ***b***) Clauss assay performed on plasma samples.

From thromboelastometric FIBTEM assay was found that Met/Met mice have an increased MCF compared to Val/Val (*Figure 13A*). Similarly, the rate of fibrinogen to fibrin conversion detected by the conventional Clauss method resulted higher in Met/Met than Val/Val mice (*Figure 13B*).

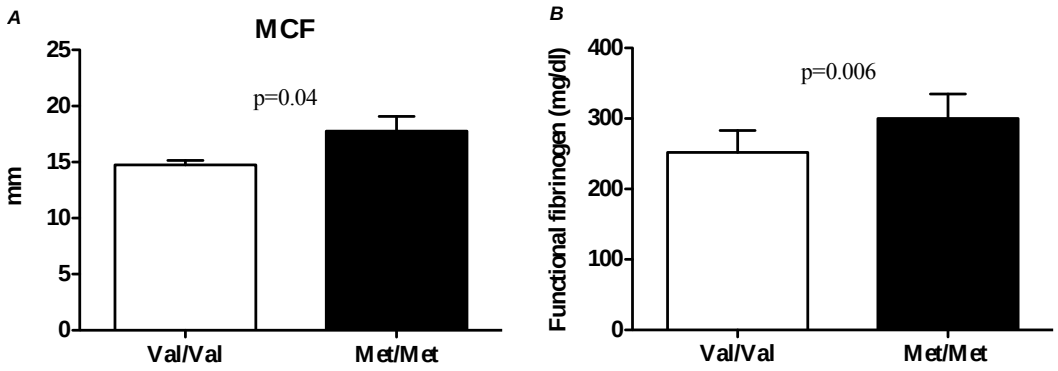

Figure 13 Functional fibrinogen. ***A***) FIBTEM test (n=8 mice per group) by thromboelastography and ***B***) Clauss test (n=11-10 mice per group) were performed on whole blood and PPP respectively of Val/Val (open bars) and Met/Met (black bars) mice to measure fibrinogen "biologically active". Data shown are mean ± SEM.

Both these results support the hypothesis that the formation and polymerization of fibrin is more considerable in knock-in mutant mice.

Finally, we measured some proteins involved in the clot stabilization and/or fibrinolytic processes. No differences were measured in terms of tissue plasminogen activator (tPA) activity, protein engaged also in pro-BDNF cleavage, and of plasminogen activator inhibitor type 1 (PAI-1) antigen and activity in plasma of Val/Val and Met/Met mice (*Figure 14A and B*). Remarkable, the tPA/PAI-1 antigen ratio was significantly lower (27%) in Met/Met mice compared to Val/Val mice, suggesting a hypo-fibrinolytic state in BDNF Val66Met homozygous mice.

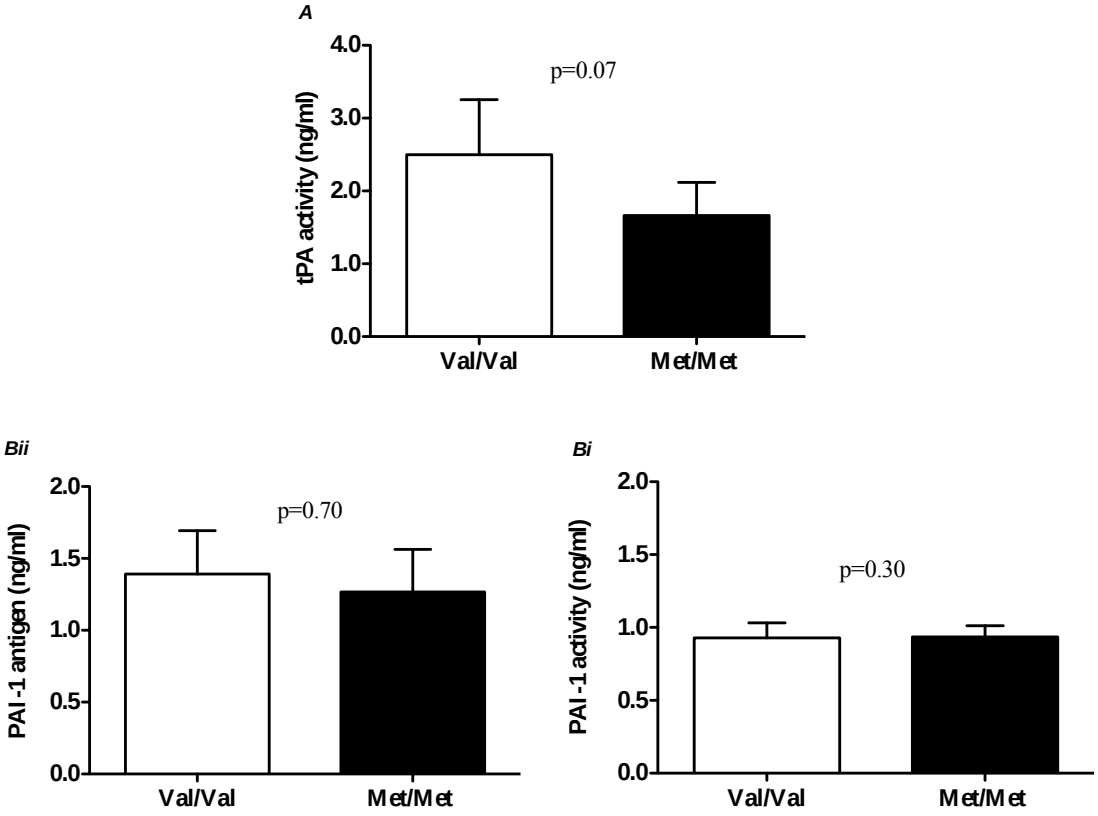

Figure 14 Fibrinolytic proteins production and activity. ***A***) tPA activity and ***Bi***) PAI-1 production and ***Bii***) activity in plasma of Val/Val (open bars) and Met/Met (black bars) mice (n=10 mice per group). Data shown are mean ± SEM.

4 Characterization of aorta tissue of Val/Val and Met/Met mice

4.1 Vessel wall proteomic analysis – Characterization of aorta tissue of Val/Val and Met/Met mice was here carried out. In particular, to determine the potential differences in aorta tissue protein in the two groups of animals, proteomic analysis of aorta secretome were performed. Aorta tissues of Val/Val and Met/Met mice were isolated, incubated for 24 hours in medium at 37°C and then supernatants were collected for characterizing the secretome. A total of 152 proteins were identified and quantified. 126 proteins were found in both genotypes, 8 were uniquely expressed in Val/Val mice and 18 uniquely in Met/Met mice (*Figure 15A*). The entire dataset was filtered by using only the identifications from LC-MS^E data set with a good replication rate (*Figure 15B*).

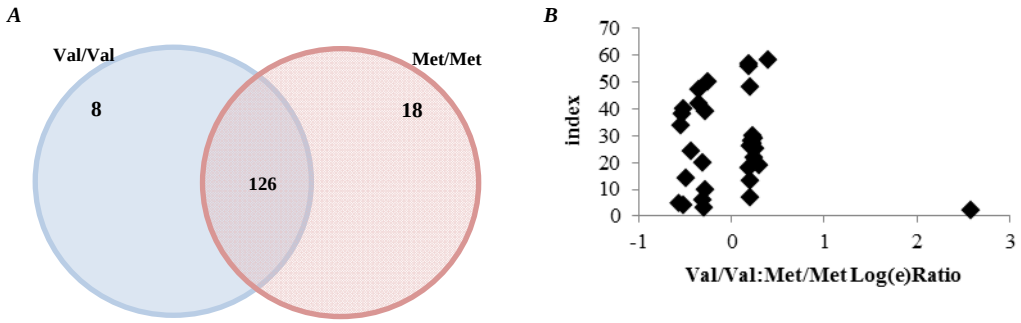

Figure 15 Proteomic analysis. ***A***) Eulero-Venn diagram for identified proteins in the secretome of aorta from Val/Val and Met/Met mice to visualize proteins expressed uniquely in one of the two groups; ***B***) Graphical representation of differentially expressed proteins between Val/Val and Met/Met mice identified by LC-MS^{E} (n=10 mice per group).

Table 3 shows the proteins identified by mass spectrometry, including those differentially expressed in two genotypes, those uniquely expressed from Val/Val or Met/Met mice, together with their fold changes (expressed as log (e) of the ratio between Val/Val and Met/Met mice), their variance and their probability.

				val/val:met/met		
Accession	**Description**	**Score**	**Unique**	**Log(e)Ratio**	**Log(e)StdDev**	**P**
Q9ERD7	Tubulin beta-3 chain	126.77	val /val			
P26443	Glutamate dehydrogenase 1, mitochondrial precursor	200.56	val /val			
Q7TMM9	Tubulin beta-2A chain	150.64	val /val			
P68372	Tubulin beta-2C chain	134.47	val /val			
P99024	Tubulin beta-5 chain	129.61	val /val			
Q922F4	Tubulin beta-6 chain	155.66	val /val			
O55023	Inositol monophosphatase	100.81	val /val			
Q9CWF2	Tubulin beta-2B chain	147.83	val /val			
P01869	Ig gamma-1 chain C region, membrane-bound form	151.37		2.58	0.29	1
Q9WVH9	Fibulin-5 precursor	392.73		0.4	0.1	1
P37804	Transgelin	1037.0		0.3	0.04	1
P58774	Tropomyosin beta chain	580.23		0.26	0.1	1
P63268	Actin, gamma-enteric smooth muscle	669.34		0.24	0.05	1
P48678	Lamin-A/C	470.81		0.24	0.15	1

P62737	Actin, aortic smooth muscle	669.64	0.23	0.06	1
P68134	Actin, alpha skeletal muscle	688.96	0.23	0.07	1
P63260	Actin, cytoplasmic 2	791.89	0.22	0.06	1
P13020	Gelsolin precursor	316.93	0.21	0.14	1
P60710	Actin, cytoplasmic 1	814.02	0.21	0.05	1
Q64727	Vinculin	2542.3 7	0.21	0.04	1
P21107	Tropomyosin alpha-3 chain	196.64	0.21	0.16	1
Q9JJU8	SH3 domain-binding glutamic acid-rich-like protein	276.41	0.19	0.18	0.98
Q9WVA4	Transgelin-2	593.15	0.19	0.09	1
P35700	Peroxiredoxin-1	192.1	0.19	0.16	0.98
Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	245.4	-0.25	0.18	0.02
Q00897	Alpha-1-antitrypsin 1-4 precursor	291.7	-0.28	0.16	0
P14152	Malate dehydrogenase, cytoplasmic	499.5	-0.28	0.09	0
P01942	Hemoglobin subunit alpha	220.36	-0.29	0.11	0
P07759	Serine protease inhibitor A3K precursor	845.63	-0.3	0.07	0
P40142	Transketolase	739.13	-0.3	0.09	0
Q03734	Serine protease inhibitor A3M precursor	565.16	-0.35	0.1	0
Q61838	Alpha-2-macroglobulin precursor	733.74	-0.35	0.11	0
P51174	Long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	436.97	-0.43	0.15	0
P22599	Alpha-1-antitrypsin 1-2 precursor	278.33	-0.49	0.12	0
P06801	NADP-dependent malic enzyme	504.28	-0.51	0.12	0
Q00898	Alpha-1-antitrypsin 1-5 precursor	622.47	-0.52	0.1	0
Q00896	Alpha-1-antitrypsin 1-3 precursor	172.36	-0.53	0.15	0
P81105	Alpha-1-antitrypsin 1-6 precursor	171.73	-0.54	0.19	0
P07758	Alpha-1-antitrypsin 1-1 precursor	172.36	-0.57	0.16	0
P42125	3,2-trans-enoyl-CoA isomerase, mitochondrial precursor	301.79	-0.66	0.21	0
P50396	Rab GDP dissociation inhibitor alpha	156.12	met/met		
P97807	Fumarate hydratase, mitochondrial precursor	150.75	met/met		
P20029	78 kDa glucose-regulated protein precursor	325.54	met/met		
Q91Z83	Myosin-7	708.41	met/met		
Q61598	Rab GDP dissociation inhibitor beta	316.08	met/met		
Q5SX40	Myosin-1	716.88	met/met		

P68369	Tubulin alpha-1 chain	160.83	met/met
Q01853	Transitional endoplasmic reticulum ATPase	337.1	met/met
P68373	Tubulin alpha-6 chain	166.79	met/met
P17182	Alpha-enolase	135.76	met/met
Q9CR68	Ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial precursor	117.84	met/met
P97427	Dihydropyrimidinase-related protein 1	130.71	met/met
P28665	Murinoglobulin-1 precursor	432.7	met/met
P32261	Antithrombin-III precursor	219.84	met/met
P13542	Myosin-8	741.74	met/met
Q60597	2-oxoglutarate dehydrogenase E1 component, mitochondrial precursor	301.08	met/met
P13541	Myosin-3	672.17	met/met
Q5SX39	Myosin-4	826.35	met/met

Table 3 Secretome analysis. Differentially expressed proteins in the secretome of aorta specimens from Val/Val mice versus Met/Met mice identified by LC–MS^E; (n=10 mice per group).

Considering biological processes in which are involved the proteins identified, we could divide them in some main families: proteins componing cytoskeleton structures and regulating cell shape (i.e. tubulins, actins, lamins and transgelin), proteins important for cell motility (i.e. myosins, tropomyosins and vinculin), protein involved in inflammatory processes (i.e. alpha-1 antitrypsin) and proteins involved in the regulation of coagulation and fibrinolytic processes (i.e. gelsolin, serine-protease inhibitors, fibulin precursor and antithrombin III precursor). In particular, we studied the impact of Met/Met polymorphism on the expression of two proteins, alpha1-antitrypsin (A1AT), involved in inflammatory processes, and gelsolin, involved in coagulation/fibrinolytic events. In addition, we analyzed the tissue factor expression and activity also in aorta tissue.

4.2 Inflammation pattern of Val/Val and Met/Met mice – The relevance of BDNF Val66Met polymorphism on inflammation, was then studied. Firstly, α_1-antitrypsin plasma levels were measured in the two groups of animals. Interestingly, A1AT was significantly higher in Met/Met mice than Val/Val mice (*Figure 16A*), confirming the results obtained by analysis of aorta secretome. Then, we determined

erythrocyte sedimentation rate (ESR) as marker of chronic inflammation. ESR test measures how fast erythrocytes fall to the bottom of a tall, thin tube. Intriguingly, ESR of Met/Met mice was increased compared to that recorded in Val/Val mice (*Figure 16B*).

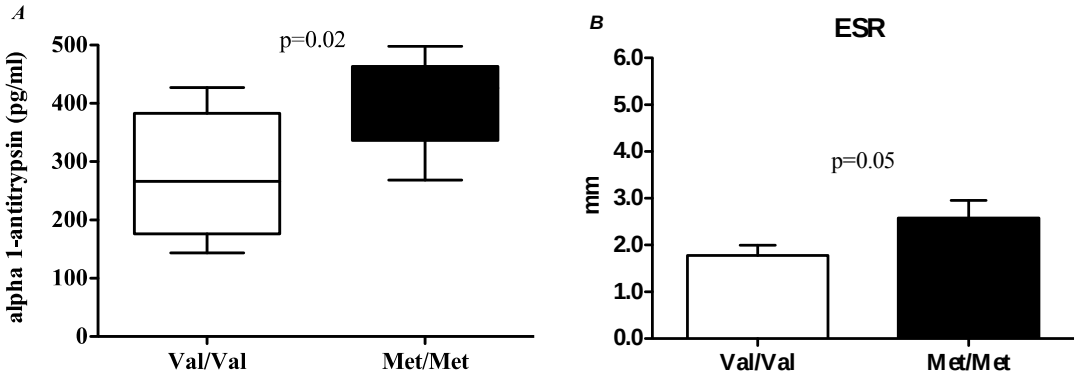

Figure 16 Plasma markers of inflammation state. ***A***) α1-antitrypsin (n=8-10 mice per group) and ***B***) erythrocytes sedimentation rate (ESR) (n=10 mice per group) were measured on plasma of Val/Val (open bars) and Met/Met (black bars) mice. Data shown are mean ± SEM.

Both these results and the higher levels of leukocyte in peripheral blood, previously shown (*Table 1*), confirm the pro-inflammatory state of Met carriers mice than control mice.

4.3 Gelsolin and Tissue Factor (TF) in Val/Val and Met/Met mice – Proteomic analysis identified gelsolin as protein differentially expressed in aortas secretome from Val/Val and Met/Met mice. Gelsolin is involved in the actin remodeling (Osborn, et al., 2006) but also in the regulation of thrombus stability (Bohgaki, et al., 2011). Consequently we measured on plasma of Val/Val and Met/Met mice the levels of gelsolin: results showed that Met/Met mice plasma contains very lower amount of gelsolin if compared to Val/Val mice (*Figure 17*)

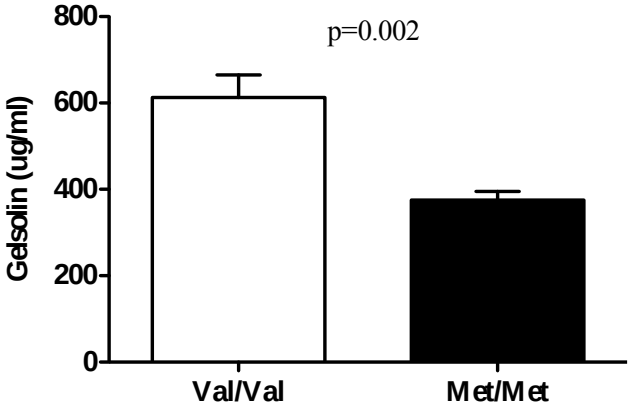

Figure 17 Plasma gelsolin. Dosage of gelsolin was performed on plasma of Val/Val (open bar) and Met/Met (black bar) mice. (n= 6 mice per group). Data shown are mean ± SEM.

In contrast, histological analysis (*Figure 18A*) and real time PCR (*Figure 18B*) showed that the expression of gelsolin was greater in aorta tissues of Met/Met mice compared to Val/Val mice.

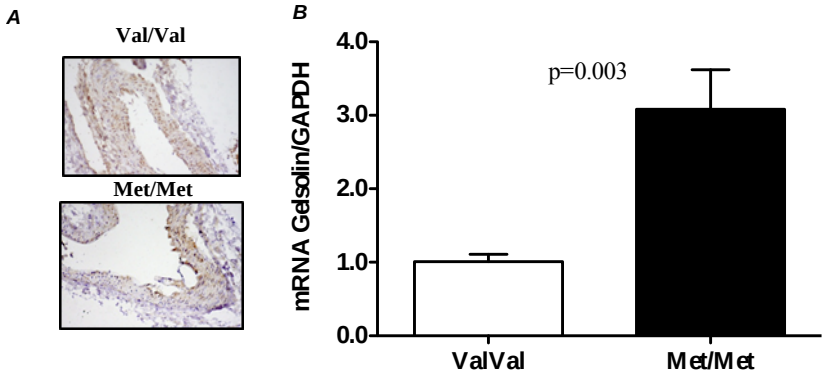

Figure 18 Gelsolin expression in aorta tissue. Gelsolin expression was evaluated by ***A***) histological analysis (immunoperoxidase staining) and ***B***) real time PCR on aorta tissues of Val/Val (open bar) and Met/Met (black bar) mice; (n=12 mice per group). Data shown are mean ± SEM.

Finally, we analyzed the expression and activity of Tissue Factor (TF) in vessel wall of the two groups of animals. Carotid arteries excised from Val/Val and Met/Met mice were lysed and processed for pro-coagulant activity (PCA) assay to measure TF activity while aortas were isolated and processed for real time PCR to measure TF mRNA expression. Vessel walls from Met/Met mice showed both higher activity (*Figure 19A*) and expression (*Figure 19B*) of TF compared to Val/Val mice.

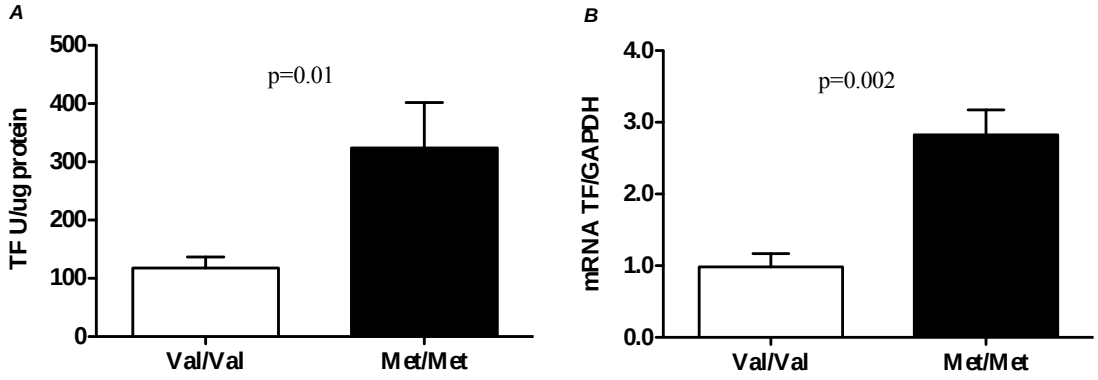

Figure 19 Tissue Factor activity and expression. TF ***A***) activity and ***B***) expression were measured in carotid artery (n=13 mice per group) or aorta tissues (n=8 mice per group) of Val/Val (open bars) and Met/Met (black bars) mice. Data shown are mean ± SEM.

5 Effect of BDNF Val66Met polymorphism on serum levels of BDNF

To assess whether the pro-inflammatory and pro-thrombotic phenotype observed in Met/Met mice is the consequence of alteration in the systemic production of BDNF we measured BDNF serum levels in Val/Val and Met/Met mice. Despite we use two specific different BDNF detection kit, the circulating levels of this neurotrophin was very low (Kit R&D and Millipore Elisa Kit). However, the results obtained using the two different Elisa kit were very similar. Indeed, the serum levels of BNDF were significantly reduced in Met/Met mice compared to Val/Val mice (*Figure 20A and B*).

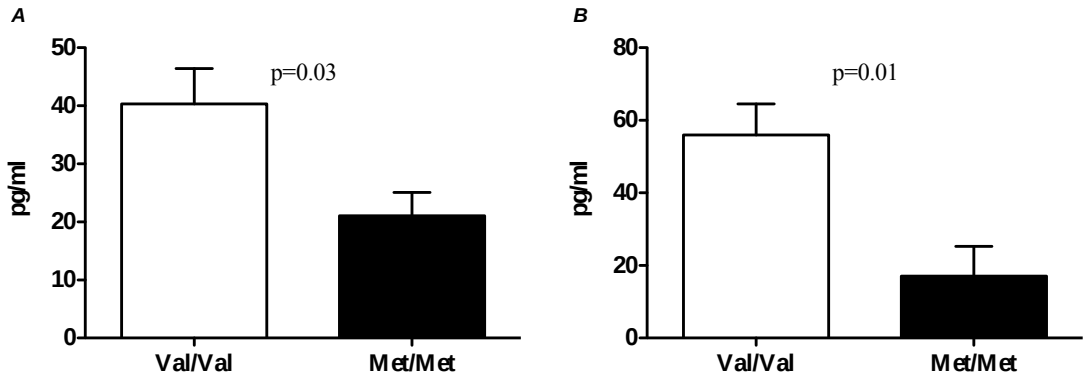

Figure 20 BDNF serum levels. Serum levels of Val/Val (open bars) and Met/Met (black bars) mice measured using ***A***) R&D Elisa Kit and ***B***) Millipore Elisa Kit (n= 6-8 mice per group). Data shown are mean ± SEM.

6 Effect of the Val66Met mutation on HeLa cells

In the last part of the study we performed some experiments to understand the real effect of the mutation Val66Met on "pro-thrombotic" phenotype observed in knock-in mutant mice. To reach this goal we transformed culture of HeLa cells with plasmids expressing the Val or Met sequence of proBDNF. By western blot analysis we visualized the correct transfection of HeLa cells, immunoblotted for C-terminal FLAG sequence, that recognizes both species of neurotrophin (pro and mature) BDNF (*Figure 21*).

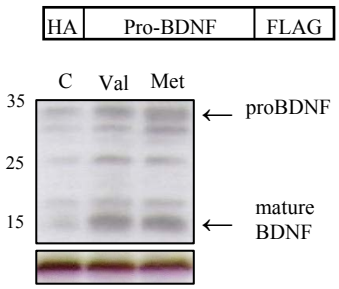

Figure 21 Plasmids constructs transfection. Dual N-terminal HA (hemoagglutinin)-tagged and C-terminal FLAG-tagged proBDNF was transfected into HeLa cells, and after 24 hours were lysated as described in Materials and Methods and immunoblotted with anti-FLAG antibodies.

Then we replicated some of the experiments carried out *ex vivo*, in particular, we analyzed levels of TF activity and gelsolin expression.

TF activity was dosed by PCA assay (*Figure 22A*) and we observed that HeLa cells expressing Met mutation showed higher TF activity, that it is translated in higher levels of TF units (*Figure 22A*). Moreover, we demonstrated that gelsolin expression, detected by western blot analysis, was strongly induced in cells transformed with Met construct, compared to lipofectamine (controls) or with HeLa transformed with Val carrying-plasmids, reaching values over 1.5 fold higher (*Figure 22B*). No difference in terms of gelsolin expression was detected between controls cells and cells transfected with plasmids expressing the Val sequence.

These results suggest that the phenotype exacerbated in our mouse model may be a direct effect of the mutation BDNF Val66Met on peripheral compartment and not only a consequence of alterations in the central nervous system.

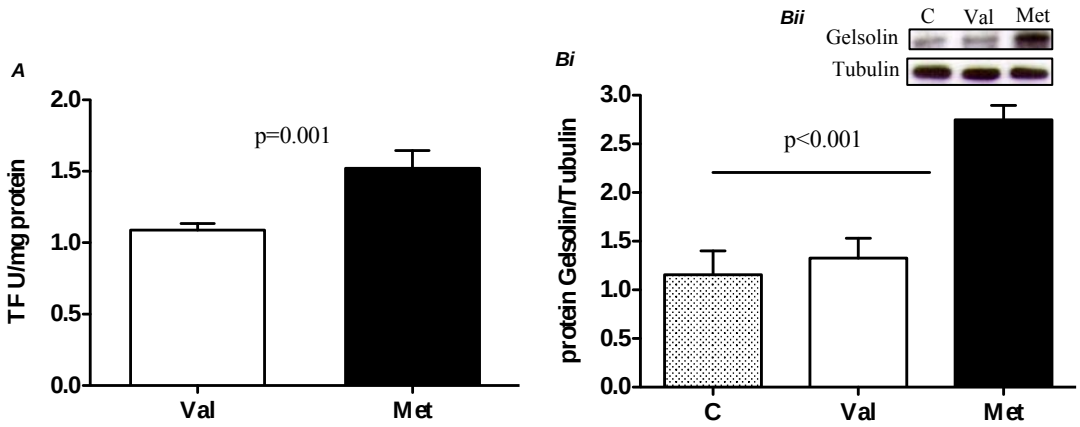

Figure 22 HeLA cells studies. HeLa cells were transformed with plasmids expressing Val (open bars) or Met (black bars) sequence; ***A***) TF activity, ***Bi***) Bar graphs summarize the changes in gelsolin protein content normalized to tubulin expression and ***Bii***) representative images of western blot. Data shown are mean ± SEM of 6 independent experiments.

4 DISCUSSION

The relevant role of neurotrophins, in particular of brain derived neurotrophic factor (BDNF), in the cardiovascular system development (Hasan, 2013) and their involvement in cardiovascular risk (Caporali, et al., 2009) (Donovan, et al., 1995) (Lorgis, et al., 2009), suggest a potential role of these neuronal mediators, in cardiovascular disease, and specifically in thrombosis.

In this study we provide evidence that genetic modification of BDNF gene, carrying the human single nucleotide polymorphism (SNP) Val66Met on the gene BDNF, exacerbates a prothrombotic and pro-inflammatory state. In particular, platelets, leukocytes and vessel wall show altered phenotype and functionality.

We observed higher thrombogenicity in mice homozygous for Met allele compared to controls, in terms of mean time of occlusion, thrombus size and percentage of mortality after the induction of different thrombosis injury models, suggesting a critical role of BDNF Val66Met polymorphism in thrombotic events.

These data may well concur with previous publications that showed that BDNF Val66Met polymorphism is independently associated with poor outcome at 2 weeks and at 1 year, and with worsening physical disability and cognitive function over that period, or with worsened surgical outcome in patients with unruptured brain arteriovenous malformation (Westbroek, et al., 2012), and that Met/Met genotype is involved in the pathogenesis of CAD in females and in the predisposition to CAD associated with depression (Bozzini, et al., 2009). Additionally, BDNF Val66Met polymorphism is associated with some cardiovascular risk factors, such as insulin resistance (Burghardt, et al., 2012) and metabolic syndrome (Zhang, et al., 2013), supporting our findings. However, a protective effect of BDNF Val66Met polymorphism on the occurrence of unstable angina pectoris (UAP) (Jiang, et al., 2009), or no correlation of this polymorphism with myocardial infarction (Stahelova, et al., 2011) were found. Several reasons can explain the different results obtained from these two studies. In particular, the different Caucasian/Asian phenotypes, in fact the genetic component of various CAD manifestations (MI and unstable angina) may partially differ. Caucasian and Asian populations differ significantly in their genetic background; this is particularly

apparent in the frequency of investigated BDNF 66Met allele in Chinese controls (49%) (Jiang, et al., 2009) by comparison with Czech controls (17%). Furthermore, the differences in haplotype structure between both ethnicities may mask causal variants within the BDNF gene or nearby. In addition, the Czech study, despite involves well-characterized group of MI patients, is disposed of lower statistical power to detect potential association of BDNF gene variant with CAD. The results of these studies should be viewed consequently with caution and need to be replicated in different population groups and settings, before a definitive relationship can be concluded upon. Moreover, the results need to be validated in a prospective study before adding this growing list of gene polymorphisms that are linked to CAD.

Our results well support the hypothesis that BDNF Val66Met polymorphism affects the physiological hemostatic processes and lead to hypercoagulable state. In fact, thromboelastographic analysis shows that whole blood of Met/Met mice forms bigger and more stable clot compared to Val/Val mice and suggests increased platelets reactivity and functional fibrinogen alteration.

In particular, higher platelet counts associated with a modest increase of reticulated platelets number were observed in Met/Met mice compared to controls. Platelets from Met/Met mice were more active as shown by higher levels of serum thromboxane B2 (TXB_2), enhanced clot retraction and aggregation, expression of membrane P-selectin and GPIIbIIIa as well as by greater ability to bind fibrinogen in response to multiple agonists.

Higher platelets counts could be partially explain the higher thrombogenicity observed in Met/Met mice, in fact platelets number may contribute to the individual risk of thrombosis (de Gaetano, et al., 2010).

In addition, reticulated platelets are more reactive platelets, aggregate more rapidly in response to collagen (Hirsh, et al., 1968), synthesize more TXA_2 (Jakubowski, et al., 1983), and express more glycoprotein (GP)Ib and GPIIb/IIIa (Tschoepe, et al., 1990). Younger reticulated platelets are significantly increased in the blood of patients with acute coronary syndromes (ACS) (Huczek, et al., 2005) (Trowbridge, et al., 1984), in diabetic patients with vascular disease (Lakkis, et al., 2004) (Huczek, et al., 2005) (Brown, et al., 1997) and predict cardiovascular death (Lakkis, et al., 2004) (Cesari, et al., 2013). Of note, increased platelet activation has also been observe in individuals prone to depression or hostility (Ehrlich, et

al., 2012) and in people subjected to high levels of work stress (Camacho, et al., 2000), suggesting how depression and CVD are strongly linked.

Activated Met/Met platelets adhere to leukocytes and form circulating mixed aggregates. The presence of increased circulating platelet-leukocytes aggregates has been observed in different cardiovascular diseases, such as stable and unstable angina, myocardial infarction and in patients undergoing percutaneous coronary interventions and heart valve replacement; additionally, platelet-leukocyte aggregates are a predictive index of acute re-occlusion following coronary angioplasty (Rezende, et al., 2013) (Buxhofer-Ausch, et al., 2012) (Cerletti, et al., 2012)

Again, the presence of platelet-leukocyte interaction is documented to be a link between inflammation, blood coagulation and vascular risk (Cerletti, et al., 2010), but also with depression. Indeed, Morel-Kopp et al. demonstrated that depression in addition to being associated with increased platelet activation and higher number of circulating CD62p (P-selectin) positive platelets, is also associated with more circulating platelet-leukocyte aggregates than controls (Morel-Kopp, et al., 2009).

In fact, elevated circulating levels of platelet-leukocyte aggregates have been reported not only in cardiac patients but also in individuals of low socioeconomic status, a factor associated with chronic psychological stress (Camacho, et al., 2000).

In our animal model increased platelet count is associated to higher leukocytes number in particular on neutrophil and monocyte populations and with pro-inflammatory markers as higher levels of α1-antitrypsin (A1AT), thrombospondin-1 (TSP-1), erythrocytes sedimentation rate (ESR) and "biologically active" fibrinogen (Gabay, et al., 1999). These data underline the relation between platelets and inflammation (Santimone, et al., 2011) that in turn constitutes cardiovascular risk (Huang, et al., 2013).

A1AT, an acute-phase reactant protein (Gettins, 2002) (Lockett, et al., 2013) plays a role in the local regulation of proteases involved in coagulation or fibrinolysis and represents a link between the inflammatory and hemostatic systems.

A strong correlation between A1AT and thrombosis has been found (Moontfoort, et al., 2013) and its plasma concentrations are increased in MDD patients (Maes, et al., 1992). In addition, BDNF Val66Met polymorphism resulted not only in a local increase expression of TSP-1 and its receptor CD36 in a post-stroke mouse model (Qin, et al., 2011), but also in the up-regulation of the circulating levels of TSP-1. TSP-1 plays an

emerging role in platelet aggregation and thrombus adhesion to injured blood vessels and limits nitric-oxide-mediated vasodilatation and tissue perfusion in experimental models of ischemic injury (Isenberg, et al., 2007). In particular, higher plasma TSP-1 levels were measured in patients with coronary artery disease and diabetes mellitus (Kyu-Young, et al., 2012).

Pavlovic et al. have demonstrated that ESR and higher fibrinogen concentration are correlated to metabolic syndrome in schizophrenic patients (Pavlovic, et al., 2013), producing another relationship among cardiovascular system, neurological disorders, inflammation and our mice model. All these findings are confirmed by several studies that propose a link between cardiovascular and depressive diseases with inflammation as common denominator (Karbach, et al., 2013) (Patas, et al., 2013).

BDNF Val66Met mice showed alteration also in the coagulation and the fibrinolytic systems. Met/Met mice showed greater Tissue Factor (TF) activity in both leukocytes and aorta tissue, while the other coagulation factors of extrinsic, intrinsic and common pathway of the coagulation cascades were not altered. TF plays a central role in our carotid artery experimental model. Indeed, ferric chloride causes significant injury both to endothelium and to the arterial media, expecting the exposure of medial TF to the circulation. The relative contributions of arterial wall-bound versus circulating TF to thrombosis remain to be determined. It was shown that circulating TF is not sufficient to produce stable thrombi, but does not rule out the possibility that it contributes to thrombus propagation (Wang, et al., 2009), consistent with a recent report showing that TF is present in pathological thrombi but not in hemostatic plugs (Hoffman, et al., 2006). Concurring with this thesis, we showed that total microparticles (MPs) TF was not significantly changed in the two groups of animals. MPs release from endothelial cells and platelets occurs by regulated budding/blebbing of the plasma membrane and is induced upon subsequent increase of intracellular Ca^{2+} (Gyorgy, et al., 2011). Since the presence of BDNF Val66Met polymorphism affects vesicles sorting and release, process favored by intracellular Ca2+ flow, (Lu, et al., 2005), we speculate that the similar total TF antigen is linked to alteration in MPs formation.

A strong but not significant decrease in the levels of tissue plasminogen activator (tPA) was detected in plasma isolated from Met/Met mice. In contrast the ratio between PAI-1 and tPA was significantly modified. Evidence suggests that tPA and plasminogen

inhibitor-1 (PAI-1) imbalance may play an important role in pathophysiology of mental and thromboembolic disorders. Indeed tPA facilitates clot dissolution and participates in several brain functions, including response to stress, learning and memory. Shi et al. showed reduction of plasma tPA and BDNF in late-onset geriatric depression patients (Shi, et al., 2010). On the other hand depression is characterized by high PAI-1 levels and is related to metabolic syndrome (Horisch-Clapauch, et al., 2013).

Of interest, in our mouse model, plasma levels of gelsolin in Met/Met mice are lower than that of Val/Val mice; on the contrary, vessel wall gelsolin mRNA and protein expression are higher compared to controls. The difference in the expression levels of two isoforms, cytoplasmic and plasmatic, is explained by different roles exerted. Plasma gelsolin clear actin filaments from the blood circulation, acting as an extracellular-scavenger system (EAAS) (Lind, et al., 1986). Cytoplasmic isoform plays roles in controlling several cellular processes such as phagocytosis (Arora, et al., 2004), motility (Kwiatkowski, 1999), apoptosis (Kwiatkowski, 1999), and platelet formation and activation (Casella, et al., 1981). The ability of plasma gelsolin to shorten actin filaments (F-actin) may therefore be of physiologic importance insofar as gelsolin-mediated diffusion of actin from the clot may restore the clot rheologic properties and makes it more sensitive to the lytic action of plasmin (Janmey, et al., 1992). In our mouse model, lower amounts of plasma gelsolin do not allow these processes, favoring the formation of a thrombus bigger and less prone to the lysis. Further investigations, in support of our observations, have demonstrated that F-actin can lead to platelet aggregation directly *in vitro*, and the presence of excessive F-actin in blood vessels, which can plug smaller vessels and decrease blood flow to promote the formation of blood clots, can be fatal (Vasconcellos, et al., 1993). In addition, gelsolin knockout mice ($Gsn^{-/-}$) display impaired function of platelets, disruption of the organization of actin-based domains in osteoclasts, alteration in the formation of growth cones of neuritis and reduced migratory capacity of neutrophils during inflammation (Witke, et al., 1995). Among several diseases in which gelsolin is altered, such as cancer, infection or inflammation, amyloidosis, Alzheimer's disease (Li, et al., 2012), there are also cardiovascular diseases. Indeed the expression of gelsolin in human heart tissues and mouse models is increased after different types of cardiac injuries, including pressure overload, dilated and ischemic cardiomyopathy,

myocardial infarction (MI), and end-stage heart failure (Li, et al., 2009) (Yang, et al., 2000) (Mani, et al., 2008).

Moreover, platelet gelsolin is highly expressed in patients with ACS (Liu, et al., 2012) and in rat after AMI (Liu, et al., 2013), and the increased levels of gelsolin in platelet reach plasma (PRP) are associated with the high platelet activation and increased level of F-actin. Decreased plasma gelsolin concentrations have been detected in plasma from AMI patients and rats, indicating that EASS is suppressed in this pathological condition (Liu, et al., 2013) (Suhler, et al., 1997). Thus, it would seem that the genetic variant BDNF Val66Met polymorphism mimics a situation of cardiovascular damage.

Finally, transfecting HeLa cells with the construct carrying or not the mutation Val66Met on BDNF gene, we reproduced some of the results obtained in animal model, supporting the hypothesis that the BDNF Val66Met mutation acts directly in peripheral cells and that what we observed *in vivo* could be due to a direct action of neurotrophin on the cardiovascular system and not only to a consequence of neuronal and innervation altered functionality.

However, it remains to clarify if the pro-thrombotic and pro-inflammatory phenotype observed in BDNF Val66Met is closely tied to the alteration in the BDNF levels. The BDNF Val66Met polymorphism, characterized by incorrect vesicles folding of mature BDNF, leads to an impairment in its neuronal release and supposedly also affects peripheral release (Lee, et al., 2009). In fact, blood BDNF concentrations reflect brain-tissue BDNF levels (Klein, et al., 2011). Studies performed on human expressing BDNF Val66Met polymorphism, showed that BDNF Val66Met homozygosity does not influence plasma BDNF levels in healthy human subjects (Luykx, et al., 2012), while there is a positive correlation between BDNF plasma levels and anxiety-like behaviors in Met carriers (Dalle Molle, et al., 2012). On the contrary, serum levels of BDNF in Met subjects, affected by mood disorders, are lower compared to controls, as demonstrated by several studies (Grande, et al., 2013) (Sotiropoulou, et al., 2013), thus suggesting effectively an altered platelets release. In fact, several studies have demonstrated that in human beings (Fujimura, et al., 2006) and in rat (Hochstrasser, et al., 2013) BDNF is stored and released in large quantities from platelets. However, the detection in plasma or serum of BDNF levels in mouse model is still controversial. Several authors showed that BDNF levels

are undetectable in mouse blood (Klein, et al., 2011), while others measure both plasma and serum levels (Borrel-Pages, et al., 2006) (Okada, et al., 2012).
Nevertheless, using two different Elisa kit, we observed a significant reduction of serum concentrations of BDNF in Met/Met mice compared to controls, suggesting that alteration on BDNF circulating levels might affect the pro-thrombotic phenotype in Met/Met mice.

We demonstrated for the first time that the genetic variant BDNF (Val66Met) polymorphism exerts effect on thrombotic events, activating the hemostatic system and altering vessel wall anti-thrombotic and anti-inflammatory physiological nature.
As previously discussed, the results here obtained are common for thrombosis, inflammation and depressive/neurological disorders. Thus, our study demonstrated that BDNF can be considered a link between cardiovascular and neurological health and pathology, activating also specific mediators of inflammation, constantly present in both cardiovascular and depressive disease.
Most importantly, our study confirmed and further supported the emerging role and direct effect of neurotrophins in cardiovascular regulation.

5 MATERIALS AND METHODS

1 Animals and *in vivo* procedures

BDNF Val66Met mice, kindly given to us by the Laboratory of Francis S. Lee, Cornell Medicine University, were generated introducing the Val66Met mutation in an ApaI-ApaI fragment containing the BDNF prodomain and put back into the targeting vector. A carboxyl terminal His tag was added by a PCR mutagenesis strategy to monitor protein expression. The loxP-Neo cassette was introduced into a EagI site as a positive selectable marker. 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 flanked by two loxP sites. Linearized targeting vectors were electroporated into 129 mouse strain embryonic stem (ES) cells. 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. Recombinant clones containing the predicted 7.4 kb rearranged band were obtained at a frequency of 1 in 40. Four positive ES clones were injected into C57BL/6 blastocysts, which were then introduced into pseudopregnant 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 (WT) mice, heterozygotes and homozygotes mice for Met/Met mutation (BDNF Val/Met and BDNF Met/Met) were generated by interbreeding heterozygotes, and offspring were genotyped by PCR analysis of tail tip-derived genomic DNA, as described (Howe, et al., 2005). All experiments were performed with 10-12 week-old animals.

1.1 Arterial thrombosis model - $FeCl_3$ injury of the carotid artery was performed as described (Mussoni, et al., 2001). Mice (gender matched) were anesthetised with ketamine chlorhydrate (75 mg/kg; Intervet) and medetomidine (1mg/kg; Virbac). The left carotid artery was dissected free. Carotid blood flow was monitored by means of a Doppler flow probe (model 0.7V, Transonic System) connected to a transonic flow meter (Transonic T106). After blood flow stabilization (baseline flow constant for 7

minutes at least 0.8 mL/sec), a 1x1 mm strip of filter paper (Whatman N°1) soaked with $FeCl_3$ (20% solution; Sigma-Aldrich) was placed over the carotid artery. After 3 minutes the filter paper was removed, the carotid artery was washed with phosphate-buffered saline (PBS), and the flow was recorded. An occlusion was considered to be total and stable when the flow was reduced by $\geq$90% of baseline within and during 5 minutes, where the flow during that 5-minute period did not change by more than 1% of baseline per second.

1.1.1 Histological analysis - Mouse carotid arteries were excised, gently perfused with PBS and fixed in 10% neutral formalin, processed, and then embedded in paraffin. Sections (5 μm) were mounted on SuperFrost® Plus microscope Slides (VWR International), and thrombi were visualized with hematoxylin and eosin (H&E) staining.

1.2 Platelet pulmonary thromboembolism - Collagen plus epinephrine-induced pulmonary thromboembolism was carried out in Met/Met and Val/Val mice. Mice were challenged with 0.1 ml of a mixture containing 480 μg/Kg collagen (MasciaBrunelli, Milan, Italy) and 60 μM/Kg epinephrine (Sigma-Aldrich), rapidly injected into one of the tail veins. The mortality of mice in each group was monitored over 15 minutes and data are presented as percentage of animals dead over total number of animals tested. At the end of each experimental session surviving animals were sacrificed by an overdose of anesthesia.

2 Blood collection

Blood samples were collected by cardiac venipuncture from anesthesized (ketamine chlorhydrate 75 mg/Kg/medetomidine 1 mg/Kg in physiologic water) Val/Val and Met/Met mice (gender-matched), into 3.8% sodium citrate (1 parts sodium citrate/ 9 blood).

2.1 Platelets rich plasma (PRP) and platelets poor plasma (PPP) – Blood samples, collected as previously described, were diluted 1:1 with Hepes-Tyrode's buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 0.35% bovine serum (BSA), 1 mM

$MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4) and centrifuged at 100g for 10 minutes. Platelet rich plasma (PRP) was gently removed with a plastic pipette and platelet counts were carried out in a Burker chamber after 1:200 dilution of the sample into Hepes Tyrode's buffer. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood at 2000 g for 20 minutes.

2.2 Serum isolation – Blood samples, collected by orbital sinus bleeding, were maintained at 37°C for 2 hours, then transferred in ice for 30 minutes. Serum was obtained by a centrifugation at 2000 g for 20 minutes.

2.3 Platelets, red blood cells and white cells counts in whole blood - Blood from gender-matched mice was collected by orbital sinus bleeding into Unopette pipettes and immediately diluted in Unopette®-System reservoirs (Becton Dickinson) containing 1% ammonium oxalate. After thorough mixing, platelets, red blood cells and leukocytes were counted optically, using the Brecher-Cronkite method, by an operator blinded to the experimental groups.

2.3.1 White cell populations count - Neutrophil granulocytes, monocytes and lymphocytes counts were performed by automatized system by BiESSE A laboratory.

3 Analysis of hemostatic system

3.1 The thromboelastography - The technology of ROTEM® is based on measurement of the viscoelastic changes that happening during coagulation *in vitro*. Thus, this technique is providing a unique possibility to evaluate initialization, formation and stability of the clot strength.

A whole blood citrated sample, isolated as previously described, is incubated in a heated cup at 37°C. Within the cup is suspended a pin connected to a detector system, that is an optical detector. The cup and pin are oscillated relative to each other through an angle of 4°75'. The movement is initiated from either the cup and the pin. As fibrin forms between the cup and pin the impedance of the rotation of the pin (ROTEM®) is detected

at the pin and a trace generated (Luddington, 2005). An analogic converter is able to translate the electromechanical signal into a signal converted by a specific software (Di Benedetto, et al., 2003). Different tests have been performed.

Recalcification time test (NATEM): To perform the test 7 µl of reagent containing $CaCl_2$ (STARTEM) 200 mM have been added to blood sample (105 µl).

Test for evaluating extrinsic pathway of coagulation (EXTEM): In this test the coagulation is triggered by a small quantity of thromboplastin (Tissue Factor-TF). To perform the test 7 µl of reagent containing thromboplastin derived from rabbit brain (EXTEM) and 7 µl of STARTEM 200 mM have been added to 105 µl of blood sample.

Test for evaluating intrinsic pathway of coagulation (INTEM): In this test the activation of contact phase is permitted by a high negative charge surface that is formed by the adding of ellagic acid and phospholipids. To carry out the test 7 µl of reagent containing ellagic acid and phospholipids (INTEM) and 7 µl of STARTEM 200mM have been added to 105 µl of blood sample.

Test for evaluating the contribute of fibrinogen to the coagulation (FIBTEM): In this test the coagulation is activated by TF and it is influenced by adding of cytochalasin D that is an inhibitor of cytoskeleton of all cells. In this way platelets contribute is excluded since it is inhibited their shape change and consequently their activation. To perform the test 7 µl of FIBTEM (reagent containing cytochalasin D) and 7 µl of STARTEM 200 mM have been added to 105 µl of blood sample.

Parameters measured: ***CT (Clotting Time)***: it is the necessary time for the formation of a clot with a diameter of 2 mm, from the beginning of the test and the addition of coagulation activator. CT is then the time in which thrombin is generated and the polymerization of fibrin begins. It is measured in seconds and it is visualized as a linear trace. CT is a useful parameter in the active state of the coagulation factors involved in the formation of thrombin, in both intrinsic and extrinsic pathway (depending on the type of activator).

CFT (Clot Formation Time): this parameter describes the kinetic of a stable clot formation by activated platelets and fibrin. It is the time between a clot of 2 mm to one of 20 mm. CFT is a measure of the necessary velocity to reach a specific level of strength of the clot and it is influenced by the number and the functionality of platelets, moreover also fibrinogen and the capacity of polymerization influenced this parameter.
MCF (Maximum Clot Firmness): it is a measure of the consistence of the clot. It is represented as the maximum amplitude obtained before that fibrinolysis reduces the clot consistence. It is expressed in mm. As MCF indicates the increasing stability of the clot by the complete polymerization of the fibrin, all cellular components (not only platelets but also erythrocytes and leukocytes) and FXIII influence this parameter.
MCE (Maximum Clot Elasticity): it is a measure of the elasticity of the clot. It is a parameter derived from MCF and it is expressed in mm. More MCE is higher more the clot is stable to the rupture.

3.2 Dosage of coagulation factors – Plasma samples, obtained as previously described, were coagulated in presence of coagulation factor deficient plasma, depending on coagulation factor analyzed. To 100 µl of tested plasma, diluted 1:10 or 1:20 in imidazole buffer (Hyphen Biomed), was added 100 µl of deficient factor plasma. After incubation of 1 minute at 37°C, TF (EXTEM, ROTEM®) and $CaCl_2$ (STARTEM, ROTEM®) were introduced and recalcification time was recorded. Recalcification time, monitored from the adding of $CaCl_2$ until the formation of fibrin clot, is determined in plastic cuvettes by manual fuss, in temperature bath at 37°C. Percentage of coagulation factor was found interpolating the clotting time on a standard curve at known concentration.

3.3 Measurement of Tissue Factor activity – ***Samples preparation.*** *Leukocytes*: leukocytes were isolated from whole blood collected as previously described, and subsequently diluted 1:2 with Tyrode Buffer. PRP was obtained as described in chapter 2 materials and methods, discarded, and the remaining fraction was diluted 1:20 with buffer for leukocytes (NH_4Cl 155 mM, $KHCO_3$ 10 mM, EDTA 0.1mM), and hold in ice for 30 minutes. Following it was centrifuged at 650 g at 4°C for 10 minutes, washed two times with PBS and centrifuged again at 1200 g for 10 minutes at 4°C. Pellet

obtained was dissolved in 150 µl of β-octil 15 mM. Later, for PCA assay, samples were sonicated for 30 seconds and hold at 37°C for 15 minutes. *Carotid artery*: carotid artery excised from Val/Val and Met/Met mice were cut, resuspended in 150 µl of β-octil 15 mM, sonicated for 30 minutes and hold at 37°C for 15 minutes before performing PCA assay. After specific protein quantification (Bradford method) samples were conveniently diluted with HEPES to obtain final protein concentration of 4 µg/40 µl and 1.25 µg/40 µl for leukocytes and carotid artery, respectively. *HeLa cells:* cells, after stimulation, were washed in PBS and lysed in 150 µl/well of β-octil 15mM at 37°C. Adherent cells were pull out with scraper and then diluted in 300 µl of HEPES 25 mM.

Pro Coagulant Activity (PCA) assay. Procoagulant activity was measured by recalcification time test of citrated mouse plasma pool at 37°C. To 40 µl of sample, pre-incubated at 37°C in temperature bath for 1 minute, were added 40 µl of PPP and 40 µl of $CaCl_2$ 15 mM. Recalcification time, monitored from the adding of $CaCl_2$ until the formation of fibrin clot, is determined in plastic cuvettes by manual fuss, in temperature bath at 37°C. Recalcification times have been converted in arbitrary units of tissue factor (TF) by using a calibration curve of thromboplastin extracted from human placenta (Behring).

3.4 Functional Fibrinogen (Clauss Method) – Blood collected, was centrifuged to obtain PPP, as previously described. PPP was then stored at -20°C till its analysis. Dosage of fibrinogen was performed by Clauss method adding to diluted sample high concentration of thrombin (100 U/ml, Sigma-Aldrich), as the clot formation is directly proportional to fibrinogen concentration present in samples analyzed (Futura System S.r.l.).

4 Platelets analysis

4.1 Platelet aggregation and adhesion - ***Platelet aggregation.*** Blood, isolated as previously described, was processed to obtain PRP. Platelet counts were adjusted to 250 x 10^3 platelets/ml with autologous PPP. Platelet aggregation was assessed by the Born

turbidimetric technique (Elvi Logos aggregometer) and was initiated by addition of various concentrations of collagen (0.175-0.5 μg/ml) or thrombin (0.0125-0.050 U/ml) and tracings were recorded for 5 minutes.

For platelets adhesion, coverlips (10 mm Ø) were coated with 0.1 mg/ml fibrinogen overnight, followed by blocking with 2% BSA for 1 hour. Washed platelets (WPs) were obtained by serial centrifugations of PRP with addition of 0.2 μM PGI_2 and 0.01 mg/L apyrase. Two-hundred μl containing 6 x 10^6 platelets were added to fibrinogen-coated coverlips and platelets allowed to adhere for the indicated time at 37°C. Wells were washed with PBS to remove non-adherent platelets, while adherent platelets were fixed with 4% paraformaldehyde for 10 minutes, permeabilized in 0.2% Triton X-100 for 5 minutes, washed with PBS, blocked with 3% BSA for 1 hour and stained with Alexafluor 488-phalloidin (1:200, Invitrogen) for 1 hour at room temperature.

4.2 Flow cytometry analysis – Flow cytometry was performed on a FACS Calibur Instrument (BD Bioscience), using CellQuest version 3.1 software (BD Bioscience), collecting 3000 events per sample.

Platelets size measurement. WPs were obtained by serial centrifugations of PRP with addition of 0.2 μM PGI_2 and 0.01 mg/L apyrase. Platelets were identified by forward and side scatter distribution, and by anti-CD41 (BD Biosciences) positivity.

Reticulated platelets. Reticulated platelets (RP) were identified by the thiazole orange method (Rocca, et al., 2002): 10 μl of PRP were incubated with 390 μl of thiazole orange (Retic-Counted, BD Bioscience) or PBS as control and anti-CD41 at room temperature for 10 minutes, in the dark, immediately after incubation, samples were analyzed by flow cytometry collecting 10,000 CD41-positive events; the percentage of RP recorded, and the absolute number of RP was calculated by multiplying by the platelet count.

Platelets-fibrinogen binding ability, GPIIbIIIa and P-selectin expression. WPs were obtained by serial centrifugations of PRP with addition of 0.2 μM PGI_2 and 0.01 mg/L apyrase. Platelet pellets were resuspended in HEPES-Tyrode's buffer at a final

concentration of 250 x 10^3 platelets/µl. Platelet activation was assessed by binding to AlexaFluor488-labeled fibrinogen (Invitrogen, Life Science/Biotechnology, Carlsbad, CA) using a PE-conjugated JON/A (Emfret Analytics, Elbestadt, Germany) antibody, raised against GPIIbIIIa (αIIβIII integrin) and with anti-CD62P, FITC-conjugated antibody (P-selectin, BD Bioscience, San Jose, CA). Twenty-five µL of WPs ($5x10^4$/uL in Hepes-Tyrodes buffer supplemented with 1 mM $CaCl_2$ and 0.35% BSA) were mixed with a saturating concentration of antibody and the mixture reacted with different concentrations of thrombin for 15 minutes at room temperature. The reaction was stopped by 400 µL ice-cold PBS, and samples were analyzed within 30 minutes.

Platelets-leukocytes aggregates. Citrated blood from Val/Val and Met/Met mice was stimulated with ADP (5 µM, Sigma-Aldrich, Italy) for 5 minute and fixed with BD lysis buffer; samples were stained with the antibodies anti-CD45 and anti-CD42c and analyzed.

4.3 Clot retraction on PRP – PRP obtained as previously described, was clotted in presence of 1 µl of autologous whole blood, recalcified with 2 mM $CaCl_2$, by adding 5 U/ml of bovine thrombin (Sigma-Aldrich). Samples were incubated at 37°C and clot retraction was recorded photographically over 150 minutes.

5 Prostanoids and plasma/serum biomarkers

TXB_2, stable metabolite of TXA_2, was measured in sera of Val/Val and Met/Met mice, by Thromboxane B_2 EIA kit (Cayman Chemical, Ann Arbor, MI).

Plasma and serum biomarkers were quantified by specific commercial ELISA kits. tPA activity: ELISA Kit for Plasminogen Activator, Tissue (tPA) (Uscn, Life Science Inc., Wuhan, Hubei, China); PAI-1 activity: Murine PAI-1 activity assay (Molecular Innovation, Inc., Novi, MI); PAI-1 antigen: Mouse PAI-1 total antigen assay ELISA kit (Molecular Innovation, Inc., Novi, MI); Thrombospondin-1: ELISA Kit for Thrombospondin 1 (THBS1) (Uscn, Life Science Inc., Wuhan, Hubei, China); α1-antrypsin: ELISA Kit for Alpha-1-Antitrypsin (α1AT) (Uscn, Life Science Inc., Wuhan, Hubei, China); Tissue Factor: ZYMUPHEN MP-TF (Hyphen BioMed); Gelsolin:

ELISA Kit for Gelsolin (GS) (Uscn, Life Science Inc., Wuhan, Hubei, China). BDNF: human BDNF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, U.S.A.) and ChemiKine TM Brain Derived Neurotrophic Factor (BDNF) Sandwich ELISA kit (Millipore, Billerica, MA, U.S.A.).

6 Erythrocytes Sedimentation Rate (ESR)

Blood samples were collected by orbital sinus bleeding in heparinized micro haematocrit tubes. Samples were immediately put vertically in plastic cuvettes and after 2 hours was measured the ratio between plasma and erythrocytes sediment. The results are expressed in mm.

7 Vessel wall analysis

7.1 Aorta secretome analysis - ***Sample preparation.*** Aorta rings were rinsed thoroughly with cold phosphate-buffered saline to remove any blood components and then were incubated in medium 199 without phenol red at 37°C for 24 hours (1 ml medium for 100 mg tissue). Conditioned media were collected, centrifuged for 5 minutes at 1000 g at 4°C to remove cell debris, and the clear supernatant was dialysed at 4°C using a 3500 molecular weight cut-off dialysis tube (Spectrum Laboratories, Rancho Dominguez, CA, USA) against 5 L of 1 mmol/L NH_4HCO_3 with two buffer exchanges at 4°C for 4 h, followed by dialysis against 0.75 mmol/L NH_4HCO_3 for 4 hours, 0.5 mmol/L NH_4HCO_3 for 16 h, 0.25 mmol/L NH_4HCO_3 for 4 hours, and a last step against distilled water for 4 hours. The samples were then concentrated by means of lyophilisation and stored at -80°C.

Preparation of protein digest. After lyophilisation, the secreted protein pellets were dissolved in 25 mmol/L NH_4HCO_3 containing 0.1% RapiGest (Waters Corporation, Milford, MA,USA), sonicated and centrifuged at 13000 g for 10 minutes. The supernatants were collected and the protein concentrations determined using Bradford's method: 45 μg of each sample were heated at 80°C for 15 minutes, reduced with 5 mmol/L DTT at 60°C for 15 minutes, and then carbamidomethylated with 10 mmol/L

iodacetamide for 30 minutes at room temperature. Digestion was performed using 1 μg of sequencing grade trypsin (Promega, Milan, Italy) overnight at 37°C. After digestion, 2% TFA was added to hydrolyse RapiGest and inactivate trypsin, and the solution was incubated at 37°C for 20 minutes before being vortexed and centrifuged at 13000 g for 10 minutes.

LC-MS analysis. Nanoscale LC separations of tryptic peptides for qualitative and quantitative multiplexed LC-MS^E analysis were performed using a nanoACQUITY system. Aortas excised from Val/Val and Met/Met mice (0.4 μg) were mixed with 50 fmol yeast alcohol dehydrogenase (ADH) digest as an internal standard for molar amount estimation (Silva, et al., 2006), and injected into a 180 μm × 2 cm Symmetry C_{18} (5 μm) trap column (Waters Corporation, Milford, MA, USA) for preconcentration and desalting. The peptide mixtures were subsequently directed from the pre-column to a 1.7 μm BEH 75 μm × 250 mm analytical column (Waters Corporation, Milford, MA, USA). The samples were eluted at a flow rate of 300 nL/minutes by increasing the organic solvent concentration from 1% to 40% B over 90 minutes, using 0.1% formic acid in water as reversed phase solvent A, and 0.1% formic acid in acetonitrile as reversed phase solvent B. All of the analyses were made in triplicate.

The precursor ion masses and associated fragment ion spectra of the tryptic peptides were measured using a SYNAPT-MS, a hybrid quadruple orthogonal acceleration time-of-flight Q-Tof mass spectrometer (Waters Corporation, Milford, MA, USA) directly coupled to the chromatographic system. The time-of-flight analyser of the mass spectrometer was externally calibrated using NaI from *m*/*z* 50 to 1990, and the data post-acquisition lock mass data was corrected using the monoisotopic mass of the doubly charged precursor of [Glu^1]-fibrinopeptide B (m/z 785.8426) delivered to the mass spectrometer at 100 fmol/μL through a NanoLockSpray interface using the auxiliary pump of a nanoACQUITY system at a flow rate of 100 nL/minutes. The reference sprayer was sampled every 30 seconds. Accurate mass data were collected in DIA by alternating low and high energy applied to the collision cell. The spectral acquisition time in each mode was 0.6 seconds, with a 0.1 seconds inter-scan delay. In the low energy MS mode, data were collected at constant collision energy of 3 eV; in high energy mode, the collision energy was ramped from 12 to 35 eV during each 0.6

seconds scan. The RF applied to the quadruple mass analyzer was adjusted in such a way that ions from *m/z* 300 to 2000 were efficiently transmitted, thus ensuring that any ion with a mass of less than *m*/*z* 300 only arose from dissociations in the collision cell.

Data processing and protein identification. The DIA data were processed and searched using ProteinLynx GlobalSERVER (PLGS) version 2.3 (Waters Corporation, Milford, MA, USA). Ion detection, data clustering, and the normalisation of the data-independent LC-MS^E data has been previously explained in detail (Geromanos, et al., 2009) (Li, et al., 2009). The proteins were identified by searching a human species-specific UniProt database (release 57.0; 20333 entries). The principle of the search algorithm has been recently described (Li, et al., 2009). The following search criteria were used for protein identification: the default search parameters included the "automatic" setting for mass accuracy (approximately 10 ppm for precursor ions and 25 ppm for product ions); a minimum of one peptide match per protein, a minimum of three consecutive product ion matches per peptide, and a minimum of seven total product ion matches per protein; up to one missed cleavage site allowed; carbamidomethyl-cysteine as fixed modification; and methionine oxidation as variable modification. The initial protein false-positive rate (FPR) of the identification algorithm was set at 4% using a randomised database that was five times larger than original database, leading to a peptide FPR that was typically smaller than 1%. However, using replication as a filter, the false-positive rate of the experiment is minimised because false-positive identifications have a random nature and do not tend to replicate across injections.

The absolute quantity of each well-characterised protein in the mixture was determined by dividing the average MS signal response of the three most abundant tryptic peptides of each protein by the signal response factor as previously described (Silva, et al., 2006).

The quantitative analyses were made using the Expression^E algorithm in PLGS 2.3 by comparing the normalised peak area/intensity of each peptide in a control and a challenged sample. The data set was automatically normalised. Briefly, in the case of complex samples, it is often possible to measure and correct for systematic errors by taking into account slight differences in protein loading amounts without using an internal standard. The assumption is that changes in protein expression occur against a dominant background of proteins that are unaffected by the studied perturbation. Each

peptide or cluster is initially treated as an internal standard by the quantification algorithm and, during this step, the peptides showing real changes are naturally suppressed as occurs in the case of inappropriate assignments or interferences in normal quantification. Subsequently, the entire data set is corrected and quantified.

Quantification has been done quantifying the peptides identified with the protein, and regulation likelihood was calculated using confidence of identification at peptide level as a quantification weighting mechanism, a method specifically designed for independently acquired LC-MS data. The overall likelihood of regulation, determined using the Quantify algorithm within PLGS 2.3 based on Bayesian statistics, is expressed by the probability of up-regulation: if this is <0.05 (i.e. between 0 and 0.05), the likelihood of down-regulation is >95%; if it is >0.95 (i.e., between 0.95 and 1) the likelihood of up-regulation is >95%.

The entire data set of differentially expressed proteins was further filtered by considering only the identifications from data with identified peptides that replicated at least two out of three technical instrument replicates. Furthermore, the significance of the regulation level was determined at 20% fold change, which is typically 2-3 times higher than the estimated error in intensity measurement (Vissers, et al., 2007).

7.2 Aorta immunohistochemistry - Immunohistochemistry analysis was performed on sections of aortas of Val/Val and Met/Met mice. Samples were fixed overnight in 4% formalin, dehydrated in a scale of alcohols and embedded in paraffin. All samples were cut in sections of 3 μm and mounted on polarized slides. Slides were rehydrated, washed in PBS containing 1% Triton X100 and treated with 3% H_2O_2 (Sigma Life Science) for 10 minutes to block endogenous peroxidase. Antigen retrieval was performed with 0.01 M citrate buffer (pH 6) in a water bath at 95°C for 10 or 30 minutes according to the antibody used. Slides were incubated overnight at 4°C with the following primary antibodies: anti-gelsolin polyclonal antibody (1:300, Novus Biologicals) or with rabbit pre-immune IgGs or goat pre-immune IgGs (Vector Laboratories, Burlingame, CA) as negative controls. After incubation with primary antibodies, sections were incubated with secondary antibodies: polyclonal anti-rabbit secondary antibody biotinylated (Dako cod. E0432) or anti-goat secondary antibody biotinylated (Dako cod. E0466) and Streptavidin/HRP (Dako cod. P0397). The reaction

was developed using 3,3-diaminobenzidine (Dako cod.K3468) as chromogen and all sections were lightly counterstained with hematoxylin. Observation and digitalization was performed by a Zeiss Axioskop (Carl Zeiss) equipped with an intensified charge-coupled device (CCD) camera system (Photometrics, Tucson, AZ).

8 Cell studies

8.1 Cell culture – Human malignant epithelial cells (HeLa) were cultured in sterile flasks and maintained in DMEM + GlutaMAX medium (Invitrogen) supplemented with HEPES (1mM, Invitrogen), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco) and 10% FBS, at 37°C in 5% CO_2/95% air atmosphere. Almost every 3 days they were moved away with trypsin (trypsin/EDTA) and transferred in new flasks after 1:4 dilution.

For transformation experiment, cells were dished on multiwell plates in DMEM + GlutaMAX medium supplement with HEPES (1mM) and FBS 10% without antibiotics. After reaching confluence of 90-95% cells were transformed with Val or Met plasmids.

8.2 Plasmids constructs – Plasmid construct were prepared as previously described (Chen, et al., 2004). Briefly, The human BDNF cDNA were subcloned into pCDNA3.1hygro expression vector (Invitrogen, Carlsbad, CA) using *HindIII* and *XhoI* sites. The HA or Flag epitope tag was added to the 3' and 5' respectively of the BDNF cDNA by PCR method. The Val to Met mutation at position 66 was generated by means of two-step PCR. Using PCR-based mutagenesis, a silent NotI site was generated immediately after the signal peptide of human BDNF to facilitate generating dual-epitope-tagged HA-BDNF-Flag construct. The HA tag was inserted just before signal peptide of human BDNF and the Flag tag was attached to C-terminal of BDNF.

8.3 Plasmids isolation, amplification, purification and transfection – ***Transformation of Escherichia Coli.*** E. coli (grown in LB Broth medium bacteria) were transformed with plasmid constructs (20 ng of plasmid/100 μl of bacterium

suspension) expressing WT or mutated sequence for BDNF (3.1 hygro HA-Val/Met-Flag) (supplied by Chen's group).

Isolation of plasmid DNA. DNA was isolated and purified by Zyppy Plasmid Miniprep Kit (ZYMO RESEARCH Corporation, Irvine, CA).

Plasmid bacterial amplification. After verifying the presence and purity of plasmids, bacterial cells expressing Val or Met construct were let to grow o/n (100 μl of bacteria in 300 μl of LB Broth base medium).

Purification of plasmids. EndoFree Plasmid Maxi Purification kit (QIAGEN, Hilden, D) was used for purification of plasmids.

HeLa Transfection. Fresh DMEM + GlutaMAX medium supplemented with HEPES (1mM), 10% FBS without antibiotics was added to cells at confluence of 90-95% and plasmids transfection was performed. Val or Met plasmids (4 μg/μl per well) were transfected in cells by lipofectamine 2000 (10 μL/well Invitrogen) and incubated for 7 hours with cells. The following day medium was changed with DMEM + GlutaMAX medium supplemented with HEPES (1mM), 10% FBS and antibiotics. After o/n incubation, cells were collected in RIPA buffer for western blot analysis or processed for PCA analysis.
Occurred transfection of plasmids was verified with western blot analysis incubating cell lysates with polyclonal Flag antibody (Sigma-Aldrich) that can detect both pro- and mature BDNF.

8.4 Protein preparation and immunoblotting - HeLa cells were lysed in cold RIPA buffer (25 mM TRIS HCl, 100 mM NaCl, 2.5 mM EDTA, 1% Triton-X-100, 0.1% SDS, 1 mM Na_3VO_4, 1 mM PMSF, 10 mM Na-pyrophosphate, 10 mM NaF pH 8.0 and protease inhibitor cocktail) and protein yield was quantified using the BCA protein assay kit (Sigma Aldrich). Thirty μg samples were prepared with the Laemmli method, and equivalent amounts of protein were separated on 7% SDS-PAGE gels, transferred to nitrocellulose membrane and bands of interest detected using antibodies

anti-FLAG (1:5000, Sigma Aldrich), anti-gelsolin (1:1000, Novus Biological),and anti-tubulin (1:10000; Sigma Aldrich) as a loading control. Subsequently membranes were incubated with peroxidase-conjugated secondary antibody, and immunoreactive bands were visualized by enhanced chemiluminescence (Thermo Scientific, Rockford, IL).

9 Real time PCR (RT-PCR)

Total cellular RNA was isolated from cells or aorta tissue with TRIzol Reagent according to the manufacturer's instructions. 1µg of RNA was reverse transcribed using SuperScript^{TM} II RNase H (Invitrogen). qRT-PCR was then carried out to assay TF, gelsolin and GAPDH was used for normalization. The primers used were:

1. ***Mouse TF***: forward 5'-CGGGTGCAGGCATTCCAGAG -3', reverse 5'-CTCCGTGGGACAGAGAGGAC-3';
2. ***Mouse Gelsolin*** : forward 5'-AGATCTGGCGTGTGGAGAAG -3', reverse 5'-CACAGTAAAGATGGCAGCAG-3';
3. ***Mouse GAPDH***: forward 5'- CGTGCCGCCTGGAGAAACC-3', reverse 5'-TGGAAGAGTGGGAGTTGCTGTTG -3'.

Samples of cDNA (2.5 µl) were incubated in 25 µL IQ Supermix containing TF, Gelsolin or GADPH primers and fluorescent dye SYBR Green (Bio-Rad Laboratories). qRT-PCR was carried out in triplicate for each sample on the CFX Connect (Bio-Rad Laboratories). Gene expression was analyzed using default and variable parameters available in the software suite provided with the CFX Connect real-time PCR Detection System (Bio-Rad). The PCR threshold cycle number (C_T) and starting quantity of test RNA samples were calculated after PCR baseline subtraction and C_T determination had been carried out on the standards. Standard curve equations were calculated by regression analysis of the log of the copy number (starting quantity) versus threshold cycle. The standard curve equations (R^2 usually >0.96) were used to calculate quantities of test RNA. The mean relative fluorescence units were calculated using individual well readings within a given PCR run on biological samples and then converted to mean fold change comparing mean relative fluorescence units of control samples versus mean relative fluorescence units of test samples.

10 Statistics - Statistical analyses were performed using GraphPad Prism 4 software. Data were analyzed by Student's *t* test or by 2-way-ANOVA (with repeated measures when necessary) for main effects of treatment time and genotype followed by a Bonferroni post-hoc analysis. P values of less than 0.05 are considered as statistically significant. Data represent mean ± SEM.

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