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# The 18 kDa Translocator Protein and Atherosclerosis in Mice Lacking Apolipoprotein E

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## 1. Introduction

### 1.1. Apolipoprotein E, inflammation and atherosclerosis

The inflammatory disease atherosclerosis is characterized by plaque formation in the cardiovascular system, which together with thrombosis can lead to obstruction of blood vessels, potentially leading to ischemia, stroke, and heart failure (Libby et al., 2009; Chen et al., 2010; Drake et al., 2011). Atherosclerosis is triggered and sustained by inflammation related cytokines, chemokines, adhesion molecules and by the cellular components of the immune system (Ross, 1999; Epstein et al., 2004). Cholesterol, most of it transported as a low density lipoprotein (LDL) particle in the bloodstream, supports foam cell formation in atherosclerotic plaques. In parallel, cholesterol plays an important role in steroidogenesis and bile production (Lacapere and Papadopoulos, 2003), which have been correlated with mitochondrial 18 kDa Translocator Protein (TSPO) and apolipoprotein E (apoE) expression (Fujimura et al., 2008; Gaemperli et al., 2011). Lipoproteins are lipid transport vehicles that ensure the solubility of lipids within aqueous biological environments. Apolipoproteins stabilize the surface of lipoproteins, serve as cofactors for enzymatic reactions, and present themselves as ligands for lipoprotein receptors. The soluble apolipoprotein gene family, which includes apoE, encodes proteins with amphipathic structures that allow them to exist at the water-lipid interface (Chan, 1989). ApoE is a polymorphic 229-aa, 34-kDa protein, which is present in the cell nucleus and cytosolic compartments (Mahley & Huang, 1999). The human gene, located on chromosome 19, encodes three alleles: apoE2 (frequency in the human population, 5–10%), apoE3 (60–70%), and apoE4 (15–20%). The isoforms differ only at residues 112 and 158 (Cedazo-Minguez & Cowburn, 2001). However, there is only one isoform of apoE in mouse and it behaves like human apoE3 (Strittmatter & Bova Hill, 2002). It is suggested that apoE deficiency in mice mimics the human apoE4 status, which implies reduced apoE3 levels relative to apoE4 levels (Buttini et al., 1999; Sheng et al., 1998).

ApoE is synthesized in several areas of the body, including the liver, where it is produced by hepatic parenchymal cells, and becomes a component in the surface of circulating triglyceride-rich lipoproteins [very low density lipoprotein (VLDL) and chylomicrons, or their remnants], and certain high density lipoprotein (HDL) particles (Mahley, 1988). ApoE plays a major role in the transport of lipids in the bloodstream, where it participates in the delivery and clearance of serum triglycerides, phospholipids, and cholesterol (Mahley, 1988). ApoE is also synthesized in the spleen, lungs, adrenals, ovaries, kidneys, muscle cells, and macrophages (Mahley, 1988). ApoE-containing lipoproteins are bound and internalized via receptor-mediated endocytosis by a number of proteins of the LDL receptor (LDLR) and LDLR-related protein (LRP) families (Davignon et al., 1998). ApoE is considered to be a ligand that binds to 27 clusters of negatively charged cysteine-rich repeats in the extracellular domains of all LDLR gene family members. It has been suggested that apoE made its entrance on the evolutionary stage long after the receptors to which it binds (Beffert et al., 2004). This also indicates that the original primordial functions of the LDLR family did not involve interactions with apoE. The original functions of the LDLR family may have been on the one hand transporting macromolecules between increasingly specialized cells and on the other hand serving as sensors for intercellular communication and environmental conditions (Beffert et al., 2003).

Cholesterol accumulation within atherosclerotic plaque occurs when cholesterol influx into the arterial wall (from apoB-containing lipoproteins) exceeds cholesterol efflux. Early in atherogenesis circulating monocytes are recruited to the arterial sub-endothelium where they differentiate into macrophages, ingest cholesterol, and develop into “foam cells” (Ross, 1973; 1999; Ross et al., 2001). Initially, monocytes adhere to activated endothelium on which up-regulated cell adhesion molecules (CAMs) are displayed, a dynamic process sensitive to inflammatory cytokines, shear stress, and oxidative insults (Chia, 1998). Induction of vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin superfamily of CAMs, is increasingly described as the key factor in monocyte infiltration (Nakashima et al., 1998; Truskey et al., 1999). ApoE-knockout mice (apoE KO) have been extensively used to study the relation of hypercholesterolemia and lipoprotein oxidation to atherogenesis (Hoen et al., 2003; Yang et al., 2009; Kunitomo et al., 2009). ApoE-deficient mice have elevated VCAM-1 in aortic lesions (Nakashima et al., 1998), which enhances monocyte recruitment and adhesion (Ramos & Partridge, 2005), while apoE expression in the artery wall reduces early foam cell lesion formation (Hasty et al., 1999). These findings imply that apoE may influence early inflammatory responses by suppressing endothelial activation and CAM expression (Stannard et al., 2001). ApoE helps protect against atherosclerosis, in part by mediating hepatic clearance of remnant plasma lipoproteins (Weisgraber et al., 1994). When apoE is absent or dysfunctional, severe hyperlipidemia and atherosclerosis ensue (Kashyap et al., 1995; Linton & Fazio, 1999). ApoE is also abundant in atherosclerotic lesions, secreted by resident cholesterol-loaded macrophages (Linton & Fazio, 1999). This locally produced apoE is atheroprotective by contributing to reverse cholesterol transport and by inhibiting smooth muscle cell proliferation (Mahley et al., 1999; Mahley and Ji, 2006). ApoE exerts several functions regarding lipid and cholesterol transport and metabolism: 1)

apoE functions as an important carrier protein in the redistribution of lipids among cells (by incorporation into HDL (as HDL-E); 2) it plays a prominent role in the transport of cholesterol (by incorporating into intestinally synthesized chylomicrons); and 3) it takes part metabolism of plasma cholesterol and triglyceride (by interaction with the LDLR and the receptor binding of apoE lipoproteins (Krul & Tikkanen, 1988; Quinn et al., 2004; Elliott et al., 2007).

ApoE has an established immune modulatory function in the peripheral immune response to bacteria and viruses (Mahley & Rall, 2000). It also modulates inflammatory responses in cell culture models *in vitro* and in *in vivo* models of brain injury, where an apoE mimetic therapeutic peptide has been shown to reduce CNS inflammation (Lynch et al., 2003; McAdoo et al., 2005; Aono et al., 2003). Involvement of apoE in injurious and inflammatory processes in the brain has attracted intensive attention (Drake et al., 2011; Potter and Wisniewski, 2012). In the brain, as well as in the cerebrospinal fluid, non-neuronal cell types, most notably astroglia and microglia, are the primary producers of apoE (Boyles et al., 1985; Quinn et al., 2004), while neurons preferentially express the receptors for apoE (Beffert et al., 2003). Regarding pathological conditions, it has been shown that human neuroblastoma cells, such as SK-N-SH, express apoE mRNA and apoE protein (Elliott et al., 2007). ApoE expression in neurons can be induced during nerve regeneration after injury and in growth and development of the CNS (Quinn et al., 2004). Moreover, Harris et al. (2004) showed that neuron-generated apoE tends to accumulate intracellularly, whereas astrocyte-generated apoE tends to be secreted. ApoE present in neurons is found in the cytoplasm (Han et al., 1994; Xu et al., 1996). The appearance of apoE in neurons may be due to neuronal synthesis under particular conditions, or by insertion into the cytoplasm of extracellular apoE (Dupont-Wallois et al., 1997). As neurons, human fibroblasts express low level of apoE under normal conditions, but under specific circumstances, such as apoptosis and nerve injury, they can produce increased levels of apoE (Do-Carmo et al., 2002; Quinn et al., 2004).

## 1.2. The 18kDa Translocator Protein (TSPO) and apolipoprotein E

Recent studies by us and others have indicated that the mitochondrial 18kDa Translocator Protein (TSPO), also known as peripheral-type benzodiazepine receptor (PBR) is present throughout the cardiovascular system and may be involved in cardiovascular disorders including atherosclerosis (Veenman and Gavish, 2006). The primary intracellular location of the TSPO is the outer mitochondrial membrane. Various studies over the course of the last 3 decennia have indicated that mitochondrial TSPO, potentially in relation to cardiovascular disease, is involved in the regulation of cholesterol transport into mitochondria in relation to bile production and steroidogenesis (Krueger and Papadopoulos, 1990; Papadopoulos et al., 2006). In particular, TSPO regulates cholesterol transport from the outer to the inner mitochondrial membrane which is the rate-limiting step in steroid and bile acid biosyntheses (Krueger and Papadopoulos, 1990; Lacapère and Papadopoulos, 2003; Veenman et al., 2007). Three-dimensional models of the channel formed by the five  $\alpha$ -helices of the TSPO indicate that it can accommodate a cholesterol molecule in the space delineated by the five helices. According to these models, the inner surface of the channel formed by

the TSPO molecule would present a hydrophilic but uncharged pathway, allowing amphiphilic cholesterol molecules to cross the outer mitochondrial membrane (Papadopoulos et al., 1997, 2006; Veenman et al., 2007). At cellular levels TSPO is present in virtually all of the cells of the cardiovascular system, where they appear to take part in the responses to various challenges that an organism and its cardiovascular system face (Veenman & Gavish, 2006), including atherosclerosis and accompanying symptoms (Onyimba et al., 2011; Bird et al., 2010; Dimitrova-Shumkovska et al., 2010a,b,c, 2012).

TSPO are located in various components of blood vessels, including endothelial cells where TSPO may take part in immunologic and inflammatory responses (Hollingsworth et al., 1985; Bono et al., 1999; Milner et al., 2004; Veenman & Gavish, 2006). To establish a factual correlation between atherogenic challenges and TSPO binding characteristics, we have previously assayed TSPO binding characteristics in different tissues of rats fed a high fat high cholesterol (HFHC) diet, in comparison to rats fed a normal diet (Dimitrova-Shumkovska et al., 2010a). It appeared that enhancement of oxidative stress in the aorta and liver due to the atherogenic HFHC diet was accompanied by significant reductions in TSPO binding density in these organs. Binding levels of the TSPO specific ligand [<sup>3</sup>H]PK 11195 in heart appeared not to be affected by the HFHC diet in this rat model.

Previous studies have shown that TSPO as well as apoE can be associated with processes such as: cholesterol metabolism, oxidative stress, apoptosis, glial activation, inflammation, and immune responses. As a ligand for cell-surface lipoprotein receptors, apoE can prevent atherosclerosis by clearing cholesterol-rich lipoproteins from plasma (Mahley and Huang, 1999). The TSPO protein has also been shown to be present in the plasma membrane of red blood cells, as well as in the plasma membrane of neutrophils, where it was shown to stimulate NADPH-oxidase activation of these cells. The plasma membrane forms of TSPO may be involved in heme metabolism, calcium channel modulation, cell growth, and immunomodulation. Furthermore, nucleus expulsion in mature erythrocytes is inhibited by excess cellular cholesterol (Fan et al., 2009). However, the involvement of the TSPO in this process has not been investigated. A recent study in cell culture showed that TSPO is important for the regulation of mitochondrial protoporphyrin IX and heme levels (Zeno et al., 2012). Thus, the TSPO appears to take part in various stages of red blood cell formation.

Furthermore, TSPO takes part in the regulation of gene expression for proteins involved in adhesion, which potentially may play a role in platelet aggregation (Bode et al., 2012; Veenman et al., 2012). ApoE has also been found to be involved in platelet aggregation, while TSPO platelet levels have been found to be increased with various neurological disorders, in particular stress related disorders (Veenman and Gavish, 2000, 2006, 2012). It has been suggested that platelet aggregation may be affected by nitric oxide (NO) generation via apoE, while other studies suggest that NO requires the TSPO to induce collapse of the mitochondrial membrane potential ( $\Delta\Psi_m$ ), mitochondrial reactive oxygen species (ROS) generation and cell death (Shargorodsky et al., 2012). Thus, the TSPO may present one pathway whereby NO does affect platelet aggregation. Furthermore, various alteration in TSPO density in the heart as a response to stress have been reported (Gavish et al., 1992; Veenman and Gavish, 2006), suggesting one aspect of involvement of TSPO in



cardiovascular diseases, including cardiac ischemia. It has also been shown that apoE is involved in cardiac ischemia (Mahley, 1988).

Apparently as a consequence of its role in steroidogenesis, TSPO typically are very abundant in steroidogenic tissues (Benavides et al., 1983; De Souza et al., 1985). Steroid hormones can affect TSPO levels, while in turn TSPO provides a modulatory function for steroid hormone production by regulation of mitochondrial cholesterol transport (Veenman et al., 2007). It is known that cholesterol affects TSPO function (Falchi et al., 2007). Interestingly, apoE is also well expressed in steroidogenic organs such as adrenal gland, ovary, and testis (Blue et al., 1983; Elshourbagy et al., 1985; Law et al., 1997). Nonetheless, studies by us suggests that elevated cholesterol levels, such as found in apoE KO mice, do not appear to affect TSPO levels in steroidogenic organs (Inbar Roim, M.Sc. Thesis, Technion – Israel Institute of Technology, 2008), even though effects in the cardiovascular system can be observed (Dimitrova-Shumkovska et al., 2010a). As has been reported, TSPO levels can be regulated by steroid hormones, which may be part of an organism's response to stress and injury (Anholt et al., 1985; Weizman et al., 1992; Gavish & Weizman, 1997; Gavish et al., 1999; Veenman et al., 2007; Mazurika et al., 2009; Veenman and Gavish, 2012). This suggests that TSPO levels may be part of a feedback control system for steroid production (responding to alterations in steroid levels), rather than be regulated by a feed forward signal provided by cholesterol (i.e. TSPO levels in relation to steroidogenesis are not being regulated by cholesterol levels *in vivo*) (Veenman and Gavish, 2012).

### 1.2.1. *Involvement of TSPO in inflammation*

Various studies have shown the presence of TSPO in all cell types of the immune system, thus proposed functional roles of the TSPO included modulation of stress-induced immunosuppression and immune cell activity (Lenfant et al., 1985; Ruff et al., 1985; Bessier et al., 1992; Marchetti et al., 1996; Bono et al., 1999; Veenman & Gavish, 2006). TSPO are present in platelets, lymphocytes, and mononuclear cells, and are also found in the endothelium, the striated cardiac muscle, the vascular smooth muscles, and the mast cells of the cardiovascular system (Veenman & Gavish, 2006). TSPO in the cardiovascular system appears to play roles in several aspects of the immune response, such as phagocytosis and the secretion of interleukin-2, interleukin-3, and immunoglobulin A (Veenman & Gavish, 2006). Mast cells are considered to be important for immune response to pathogens (Marshall, 2004) and they have also been implicated in the regulation of thrombosis and inflammation and cardiovascular disease processes such as atherosclerosis as well as in neoplastic conditions (Wojta et al., 2003). Studies have shown that benzodiazepines' inhibition of serotonin release in mast cells could reduce blood brain barrier permeability, influence pain levels, and decrease vascular smooth muscle contractions (Veenman and Gavish, 2006). Benzodiazepines have been found to bind to specific receptors constituted by the TSPO on macrophages and to modulate *in vitro* their metabolic oxidative responsiveness (Lenfant et al., 1985). TSPO in the cardiovascular system also has been associated with the development of atherosclerosis (Camici et al., 2012). It for example has been suggested that reductions in TSPO levels may act as a protective mechanisms against the development of oxidative stress in aorta and liver (Dimitrova-Shumkovska et al., 2010a, b, c; 2012).

Anti-inflammatory properties of TSPO ligands have been demonstrated in various tissues. TSPO ligands have been shown to reduce inflammation in animal models of rheumatoid arthritis (Waterfield et al., 1999), carrageenan-induced pleurisy (Torres et al., 2000), and pulmonary inflammation (Bribes et al., 2003). Taupin et al. (1993) have also demonstrated *in vivo* that the synthetic TSPO ligand Ro5-4864 increases brain IL-1, IL-6 and TNF- $\alpha$  production after brain trauma. These cytokines are known to play a role in the inflammatory reaction to brain injury (Heumann et al., 1987). Interestingly, one study showed that PK 11195, but not Ro5-4864, could exert anti-inflammatory actions on mononuclear phagocytes, regulating the release of IL-1b (Klegeris et al., 2000). In addition, *in vivo* studies have shown that TSPO ligands can reduce the typical inflammatory response presented by reactive microglia and reactive astroglia resulting from brain trauma (Ryu et al., 2005; Veiga et al., 2005).

### 1.3. Animal models and strategies for atherosclerosis study

Atherosclerotic plaques may appear early in life and might progress into severe, symptomatic plaques many decades later, dependent on the coexistence of risk factors such as age, genetic background, gender, hypercholesterolemia, hypertension, smoking, diabetes, etc. (Ross, 1999; Whitman, 2004). Rupture of lipid-rich coronary plaques can trigger an atherothrombotic event and probably is the most important mechanism inducing acute coronary syndrome (ACS) (Vilahuer et al., 2011).

Plaque rupture presents a major factor in ischemic processes associated with atherosclerosis (Zhao et al., 2008; Cheng et al., 2009; Gaemerli et al., 2011). Plaque rupture in the human condition, including the cardiovascular processes and events leading up to it, presently is virtually inaccessible for research. Therefore, animal models have been developed to study atherosclerosis, including plaque rupture and thrombus formation, and also how to take measures to prevent these from happening. Nonetheless, more sophisticated models need to be developed and tested to be able to better mimic the human condition. This is so, as mice and rats, for example, do not develop atherosclerosis without genetic manipulation, because they have a lipid physiology that is radically different from that in humans, as most of the cholesterol is being transported in HDL-like particles (Whitman, 2004; Singh et al., 2009; Vilahur et al., 2011). Furthermore, all of the existing animal models, including biological and mechanical triggering of atherogenesis, e.g., the Watanabe heritable hyperlipidemic (WHHL) rabbit model, the apolipoprotein E (ApoE) mouse model, and the LDL-receptor mouse model) suffer the drawback of lacking an end-stage atherosclerosis that would show plaque rupture accompanied by platelet and fibrin-rich occlusive thrombus at the rupture site (Singh et al., 2009). Another restriction of current models for cardiovascular disorders is that most of the studies explore only male mice to avoid effects of estrogens to the extent of lesion development and diminishing LDL oxidation (Caligiuri et al., 1999; Yang et al., 2004). As cardiovascular disorders also occur in women, it would be valuable to also study female animal research subjects. Furthermore, it would give direction to research relating hormonal conditions to atherosclerosis.

Cholesterol lowering by diet is associated with a reduction in DNA damage, at least in animal models (Singh et al., 2009). In general, modification of atherosclerotic risk factors by lipid lowering therapies, cessation of smoking, weight loss, and improved glucose control reduces circulating markers of inflammation. These and other findings suggest that inflammation is a primary process for atherosclerosis (Ziccardi et al., 2002; Rodriguez-Moran et al., 2003). Although high dietary intake of the anti-oxidant vitamin E and C has been associated with reduced risk of cardiovascular disease (CVD), well powered clinical trials in atherosclerosis-related CVD have indicated that supplements with vitamin C or vitamin E alone do not provide sufficient benefit, in comparison to, for example, statins (Kunitomo et al., 2009). Furthermore, specific antioxidants scavenge or metabolize some, but not all of the relevant oxidized molecules (Stocker and Keaney, 2004). Stocker and Keaney (2005) conclude that whenever a physiological process goes unchecked in case of disease, treatment strategies cannot simply rely on scavenging ROS. Nonetheless, drugs that have been proven to alter plaque progression have also been shown to alter vascular oxidative stress. For example, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA) inhibitors (Statins) reduce NAD(P)H oxidase activation and superoxide production *in vitro*, in part because of their capability to inhibit the membrane translocation (and thus activity) of the small GTP-binding protein Rac-1, which is a regulatory component of vascular NAD(P)H oxidase activation (Costopoulos et al., 2008). In conclusion, it appears that beneficial therapeutic treatments to prevent atherosclerosis include lowering of lipid levels and also reduction of oxidative stress. However, restricting a treatment to only reduction of oxidative stress does not appear to generate sufficient beneficial effects to counteract atherosclerosis.

## **2. The effects of cholesterol challenges that result in atherogenesis on TSPO binding density in aorta and heart**

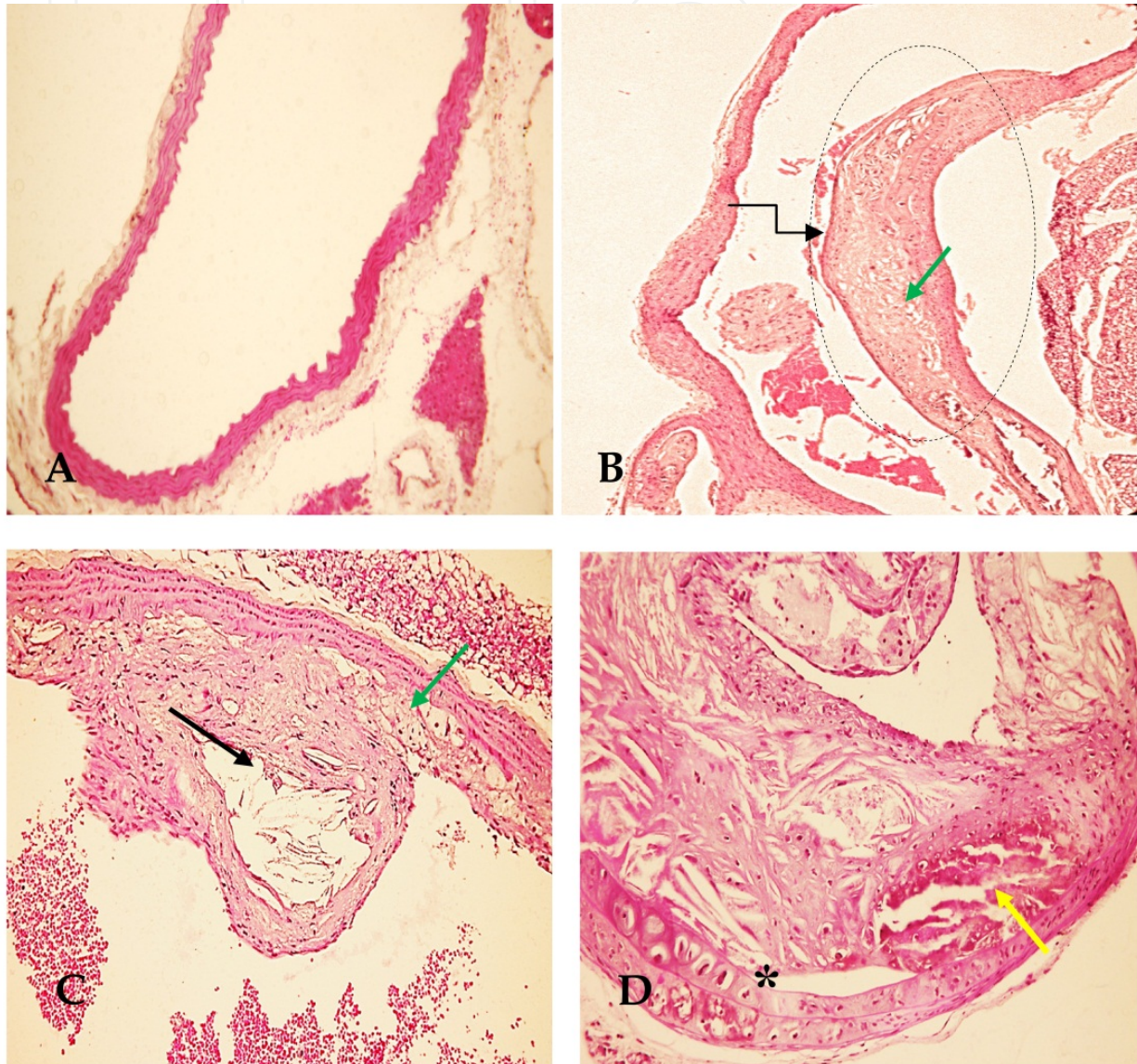
As apoE deficiency may increase cholesterol levels and induce NO generation, which in turn may affect TSPO function, we were interested to study whether TSPO binding characteristics may be affected in heart and aorta of apoE-knockout (B6.129P2-apoE<sup>tm1</sup> N11) mice, in comparison to their C57BL/6 background mice (i.e. wild type, WT). For the present study homogenates of whole heart organ and aorta segments (aortic arch and descending aorta) were used. For this approach, it was taken into consideration that accumulation of proatherogenic lipid affects all cells types present into vascular wall, and the response of the entire tissue to the cholesterol exposure is relevant as an indication of vascular defense as a whole (Hoen et al., 2003). All procedures with the animals were in accordance with National Institutes of Health (USA) guidelines for the care and use of experimental animals (NIH publication No. 85-23, revised 1996), and the experimental protocol was reviewed and approved by the local ethics committee. The mice were housed in polycarbonate cages in a pathogen – free facility set on a 12h light-dark cycle and given *ad libitum* access to water and standard laboratory feed. Prior to the experimental procedures, the rats were fed a commercial standard pellet feed (Filpas, 52.11, Skopje, Republic of Macedonia), named “standard feed” hereafter.



At 16 weeks of age, animals were randomized into experimental groups: i) Two control groups (WT mice,  $n = 10$ ) and (apoE KO mice,  $n = 10$ ), both these control groups received standard feed for a additional period of 10 weeks; ii) Two experimental groups receiving the same feed for the same 10 weeks but supplemented with 1% cholesterol (1% WT mice,  $n = 10$ ) and (1% apoE KO mice,  $n = 10$ ); and iii) Two experimental groups received the same feed for the same 10 weeks but supplemented with 3% cholesterol (3% WT mice,  $n = 10$ ) and (3% apoE KO mice,  $n = 10$ ). After these 10 weeks, animals were sacrificed by cardiac puncture, under ketamine/xylazine anaesthesia, followed by the appropriated storage until application or procedures required for assays of TSPO binding characteristics, ROS parameters, and histopathology, as described in detail previously (Dimitrova-Shumkovska et al., 2010 a, b, c, 2012). Tissue homogenates of aorta and heart were prepared for our various assays. For TSPO binding assays, tissue homogenates were prepared in 50 mM PBS on ice with a Kinematika Polytron (Luzerne, Switzerland), as described previously (Dimitrova-Shumkovska et al., 2010 a, b, c). To prepare homogenates for assays of oxidative stress parameters, we used an Ultrasonic Homogenizer (Cole-Parmer Instrument Co., Chicago, IL) as described previously (Dimitrova-Shumkovska et al., 2010 a, b, c). For advanced oxidation protein products (AOPPs, Witko-Sarsat et al., 1996), tissue homogenates were prepared in 50 mM PBS at + 4 °C, as described previously (Dimitrova-Shumkovska et al., 2010 a, b, c). For the other assays of oxidative stress (see below), tissue homogenates were prepared in 1.12 % KCl at + 4 °C, as described previously (Dimitrova-Shumkovska et al., 2010 a, b, c). These later parameters of oxidative injury included: lipid peroxidation products [TBARs] (Draper and Hadley, 1990); protein carbonylation, PC (Shacter, 2000); superoxide dismutase activity (SOD assay kit, RA20408, Fluka, Biochemika, Steinheim, Germany), glutathione (GSH assay kit CS0260, Sigma-Aldrich, Steinheim, Germany), glutathione reductase (GSSG-Red), GRSA 114K4000, Sigma-Aldrich, Steinheim, Germany], Finally, aortas were prepared for anatomical observation and histopathology as described previously (Dimitrova-Shumkovska et al., 2010 a, b, c).

Effects of cholesterol supplements to the apoE KO mice on plaque formation in the aorta are shown in Figure 1. No atherosclerotic formation was found in WT mice regardless of diet (Figure 1A). Control aortas of apoE KO mice having access to standard feed are characterized by the presence of thin fibrous tissue caps i.e. encapsulations of collagen rich fibrous tissue without a necrotic core that showed only superficial accumulation of foam cells (Figure 1B). Cholesterol diet accelerated atherosclerosis in apoE KO mice, increasing the total surface area of plaque formation significantly over the intimal area (Figure 1C) compared to apoE mice receiving standard feed. In 1% cholesterol fed apoE KO mice, expansion of the necrotic core presenting an important pathogenic process contributing to plaque vulnerability was observed in comparison to standard fed apoE mice (Figure 1C). After administration of 3% cholesterol diet to apoE KO mice even more advanced lesions have developed. Initial xanthoma formation, cartilage tissue, and calcified nodules with an underlying fibrocalcific plaque with minimal or absence of necrosis occurred (Figure 1D). Furthermore, plaques become more progressive and lesions show luminal stenosis with pathologic intimal thickening. These observations are in line with other research data, where plaque rupture was seen in apoE KO mice especially when exposed to western type diet

(Davignon et al., 1999; Johnson et al., 2005). ApoE KO mice can also develop interplaque hemorrhage and features of plaque instability that are accelerated by feeding westernized diet (Rosenfeld et al., 2000). "Western type diets for mice" typically utilize just one ingredient (milk fat or lard) as the primary source of energy from fat.



**Figure 1.** Representative cross-sections of mice aortas. A) No atherosclerotic lesions were found in wild-type mice regardless of the diet; B) atherosclerotic plaque (outlined) characterized by a thin fibrous tissue cap (elbow black arrow), particularly superficial accumulation of foam cells (green arrow) without a necrotic core and encapsulated by collagen rich fibrous tissue in apoE KO mice given standard feed; C) accelerated atherosclerosis and deposition of cholesterol crystals (black arrow) in the endothelium of the aorta wall in 1% apoE KO; D) advanced lesions are developed in 3% apoE mice. Initial xanthoma formation, cartilage tissue (asterisk) and calcified nodules (yellow arrow) with an underlying fibrocalcific plaque with minimal or absence of necrosis occur (H&E staining, microscopic magnification applied x 100).

Plasma lipoprotein levels mg/dL										
Strain		Chol			TAG			HDL		
	wk	C	1%	3%	C	1%	3%	C	1%	3%
WT	16	67.7 ± 23.3	88.07 ± 28.0	110.0 ± 27.0**	71.0 ± 18.4	71.5 ± 5.1	97.5 ± 16.2**	33.2 ± 5.1	33.6 ± 10.8	74.7 ± 10.8***

Plasma lipoprotein levels mg/dL										
Strain		Chol			TAG			HDL		
	wk	C	1%	3%	C	1%	3%	C	1%	3%
Apo E <sup>(-/-)</sup>	16	383.7 ± 47.3	457.23 ± 62*	555.4 ± 83.3***	117.7 ± 24.5	105.6 ± 11.4	159.4 ± 59.0**	67.0 ± 37.5	66.6 ± 16.3	32.9 ± 11.4**

**Table 1.** Effects of cholesterol (Chol) supplemented diet for 10 weeks, on lipoprotein levels in apoE KO mice and their WT counterparts. Unpaired Student *t*-test was performed. Data are expressed as mean ± SD; \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

Changes in the serum levels of total cholesterol, triglycerides and HDL-cholesterol in each group are shown in **Table 1**. Corroborating previous studies (Davignon et al., 1999; Seo et al., 2005; Zhao et al., 2008) at 16 weeks of age, even before application of the cholesterol enriched diets, apoE KO mice, already displayed approximately 5 times higher levels of total cholesterol in comparison with WT mice. At this time point, no significant differences in triglycerides (TAG) levels were observed between WT mice and apoE KO mice. However, 3% diet regimes, caused significant increases in total cholesterol level in apoE KO mice (by 44%,  $p < 0.001$ ), compared to standard feed. The enhanced total cholesterol levels, included an almost 90% representation of non HDL – cholesterol (calculated from Friedewald formula; Friedewald et al., 1972). In contrast, 3% WT mice, showed significantly higher cholesterol levels (by 62%,  $p < 0.01$ ), including an almost 70% representation of HDL-lipoproteins. Supplement of 3% cholesterol also provoked significantly higher triglycerides levels: by 35 % ( $p < 0.01$ ) in apoE mice and by 36% ( $p < 0.01$ ) in WT mice. Supplement of 1% cholesterol, resulted in slight increases in total cholesterol in apoE mice (by 20%,  $p < 0.05$ ), but did not significantly affect the triglycerides levels. The same type of diet did not affect lipoprotein levels in WT mice.

In the aorta, 3% cholesterol diet supplement, caused significant increases in “steady-state” levels of lipid peroxides (TBARs) and oxidized proteins in WT as well as apoE KO mice (**Table 2**). In detail, regarding lipid peroxidation, TBARs production was significantly increased by 2 fold in WT and apoE KO mice subjected to 3% cholesterol supplemented diet (+100%,  $p < 0.01$  for WT mice, and +125%,  $p < 0.001$  for ApoE KO mice). In parallel, protein oxidation products levels (AOPP) were also significantly higher (+135%,  $p < 0.01$  in 3% WT mice and +177%,  $p < 0.001$ , in 3% apoE KO mice). Protein carbonyls (PC) showed a slight but non-significant increase in 3% cholesterol fed WT and apoE KO mice, compared to their controls. In contrast to the 3% diet regime, 1% cholesterol supplemented diet did not affect ROS parameters in aortic tissue in both WT and apoE KO mice.



Variables/ Aorta	WT Control	1% Chol	3% Chol
TBARs nmol/mg	0.16 ± 0.04 (n=8)	0.17 ± 0.06 (n=7)	<b>0.32 ± 0.08** (n=8)</b>
AOPP nmol/mg	37.1 ± 11.3 (n=7)	44.1 ± 19.3 (n=8)	<b>86.8 ± 21.4** (n=8)</b>
PC pmol/mg	45.7 ± 11.0 (n=7)	55.2 ± 22.8 (n=8)	55.3 ± 11.8 (n=8)

Variables/ Aorta	Apo E Control	1% Chol	3% Chol
TBARs nmol/mg	0.24 ± 0.07 (n=8)	0.29 ± 0.11 (n=8)	<b>0.54 ± 0.25*** (n=10)</b>
AOPP nmol/mg	22.0 ± 17.1 (n=8)	27.9 ± 7.1 (n=8)	<b>60.5 ± 30.6*** (n=10)</b>
PC pmol/mg	46.6 ± 20.3 (n=8)	45.3 ± 11.3 (n=8)	52.1 ± 10.6 (n=12)

**Table 2.** Effects of cholesterol (Chol) supplemented diet for 10 weeks on aorta oxidative stress parameters in apoE KO mice and their WT counterparts. 1-way ANOVA followed by application of the Tukey test to assess the significance of specific intergroup differences. Data are expressed as mean ± SD; \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

The capacity of glutathione as an electron donor to regenerate the most important antioxidants (vitamin E, glutathione peroxidase (GPx), lipid hydroperoxides), is linked with the redox state of the glutathione disulfide – glutathione couple GSSG/2GSH (Schafer and Buettner, 2001). This in turn, has a high impact on the overall redox environment in the cell. Concerning antioxidant activities in aorta tissue due to 3% cholesterol supplemented feed, significantly reduced activity of superoxide dismutase (SOD) was measured in 3% apoE KO mice compared to standard feed mice (- 41%, **Table 3**). The results also suggest a significant reverse interaction between glutathione level (GSH) and glutathione peroxidase (GPx) activity in aorta tissue. In particular, the analyzed results indicated that the glutathione content in aorta of 3% apoE animals was significantly decreased (-32%), with simultaneous slight, but significant enhancement achieved in activity of glutathione peroxidase (+10%), as compared to standard feed control ( $p < 0.05$ ). In parallel, glutathione content in aorta was also significantly reduced in 3% WT mice for 70% ( $p < 0.01$ ), without affecting GPx levels. Feeding the mice diet supplemented with 1% cholesterol, resulted in significantly reduced activity in SOD in apoE KO mice (by 33%  $p < 0.05$ ) and in WT mice (by 47%  $p < 0.05$ ).

To determine TSPO binding characteristics in this paradigm we applied binding assays with the TSPO specific ligand [ $^3\text{H}$ ] PK 11195. The present study sought to determine whether cholesterol supplementation affects TSPO binding characteristics in aorta and heart of apoE KO mice in association with parameters for oxidative stress. Binding assays of the heart and

Variables/ Aorta	WT Control	1% Chol	3% Chol
SOD U/mg	4.76 ± 1.5 (n=9)	2.5 ± 0.9* (n=7)	1.45 ± 0.6** (n=7)
GSH nmol/mg	7.8 ± 4.2 (n=9)	8.3 ± 3.7 (n=9)	3.7 ± 0.8** (n=9)
GPx mU/mg	0.261 ± 0.01 (n=8)	0.273 ± 0.01 (n=8)	0.257 ± 0.03 (n=8)

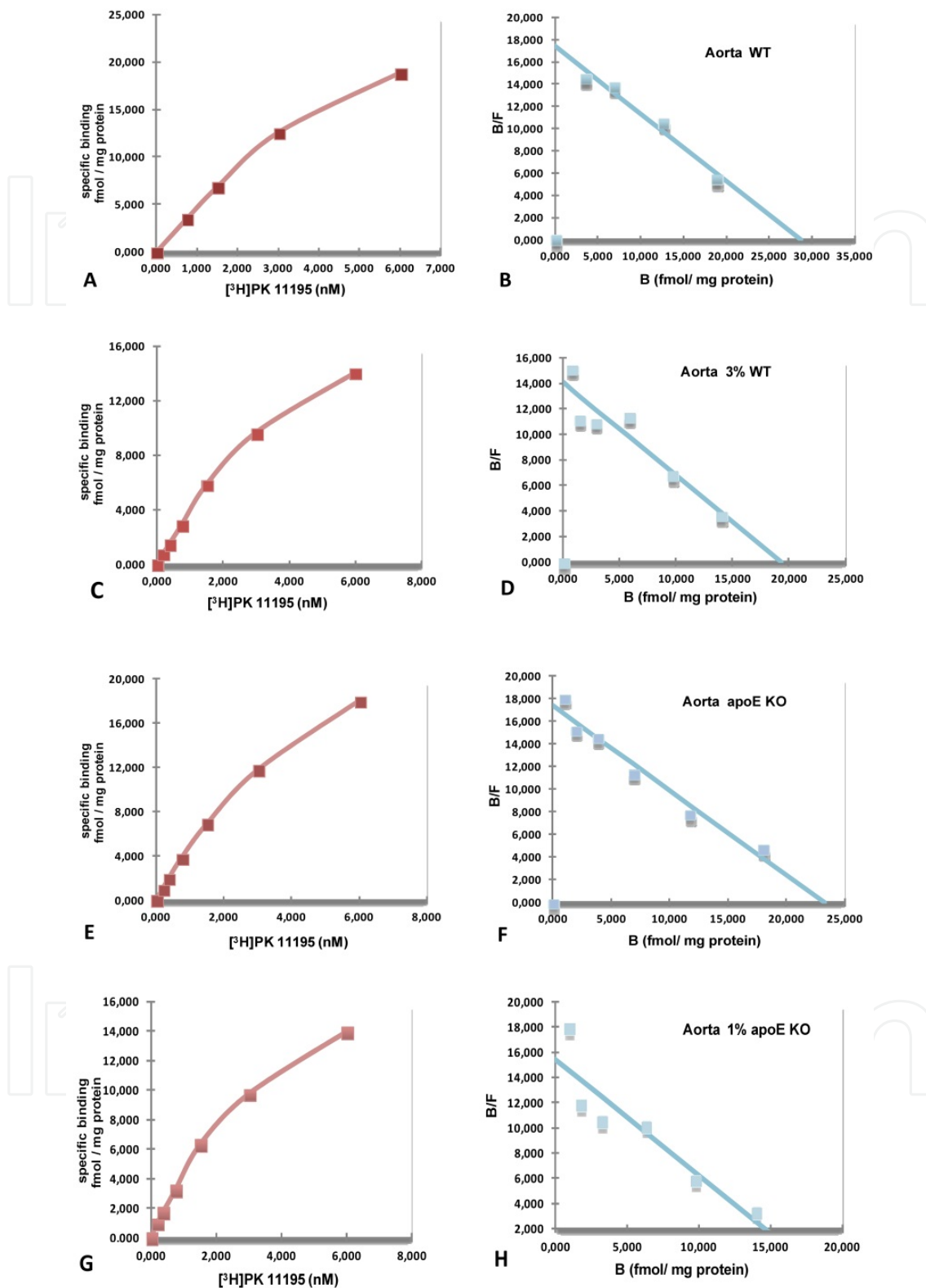
Variables/ Aorta	Apo E Control	1% Chol	3% Chol
SOD U/mg	6.87 ± 1.6 (n=7)	4.6 ± 1.1* (n=6)	4.05 ± 0.88* (n=8)
GSH nmol/mg	6.7 ± 2.9 (n=9)	7.4 ± 2.7 (n=9)	4.5 ± 2.0* (n=9)
GPx mU/mg	0.242 ± 0.02 (n=9)	0.256 ± 0.01*(n=7)	0.266 ± 0.03* (n=7)

**Table 3.** Effects of cholesterol (Chol) supplemented diet for 10 weeks on aorta antioxidant parameters in apoE KO mice and their WT counterparts. Unpaired Student *t*-test was performed. Data are expressed as mean ± SD; \* = *p* < 0.05, \*\* = *p* < 0.01.

aorta with the TSPO specific ligand [<sup>3</sup>H]PK 11195 were done to determine potential effects of cholesterol supplementation on TSPO binding characteristics, according to methods described previously (Dimitrova-Shumkovska et al., 2010 a,b,c). For representative examples, see **Figure 2**. In heart, only in WT mice significant decreases in the B<sub>max</sub> of TSPO (- 42%, *p* < 0.001) was determined with [<sup>3</sup>H]PK 11195 binding as a consequence of both cholesterol 1% and 3% supplemented diets, compared to control standard fed WT mice. Regarding the apoE KO mice, cholesterol supplemented diet did not induce differences in the TSPO binding characteristics in the heart (**Table 4**). Regarding heart tissues, both in the apoE KO groups and WT groups, K<sub>d</sub> values determined with [<sup>3</sup>H] PK 11195 binding were in the nM range (0.6 – 1.6 nM) showing no significant differences between experimental and control groups.

Regarding the aorta, feeding the mice with standard feed was not accompanied by significant differences in the TSPO binding characteristics of the aorta of apoE KO mice versus WT mice (**Table 4**). Interestingly, these mouse aortas showed very TSPO binding levels, comparable to those observed in the adrenal of rats (Gavish et al., 1999). To date, the adrenal of rats is the tissue with one of highest demonstrated B<sub>max</sub> for TSPO ligand binding (Gavish et al., 1999). The 1% cholesterol supplemented diet significantly reduced TSPO binding capacity in aorta in both WT and apoE KO mice. In particular, reductions by 49% in WT mice and by 32% in apoE KO mice (*p* < 0.001 and *p* < 0.01, respectively) compared to their standard feed controls were observed (**Table 4**). The 3% cholesterol diet also provoked a reduction in TSPO binding density by 58% in the aorta (*p* < 0.01), but only in WT mice. In the aortas of both groups, apoE KO mice and WT mice, K<sub>d</sub> values determined with [<sup>3</sup>H] PK 11195 binding were in the nM range (1.5 – 2.6 nM), showing no significant differences between the groups.





**Figure 2.** Representative examples of saturation curves (A, C, E, G) and their Scatchard plots (B, D, F, H) of  $[^3\text{H}]\text{PK 11195}$  binding to membrane homogenates of aorta, respectively of WT mice (A, B, C, D) and apoE KO mice (E, F, G, H). Abbreviations: apoE KO = apolipoprotein deficient mice; WT- wild type mice; B: bound; B/F: bound over free.

As the effects on TSPO binding density in heart and aorta due to intake of cholesterol supplemented diet take place primarily in the WT groups, and especially not in the 3% cholesterol diet fed apoE KO mice, these data suggest that decreases of TSPO binding density in heart and aorta may serve to counteract processes typically leading to cardiovascular damage, including atherosclerosis, as explained in more detail in the Discussion.

Bb - wild type mice									
Bb - Control				Bb - 1% Chol.			Bb - 3 % Chol.		
Tissue	B max (fmol/mg)	Kd (nmol)	n	B max (fmol/mg)	Kd (nmol)	n	B max (fmol/mg)	Kd (nmol)	n
Heart	1740 ± 180	0.65 ± 0.1	5	1005 ± 240**	1.12 ± 0.3	6	1006 ± 140 **	1.32 ± 0.5	5
Aorta	29 000 ± 9700	2.50 ± 1.2	9	14 900 ± 3370***	2.31 ± 0.8	6	12 200 ± 2920**	2.62 ± 0.5	5

Apo E KO mice									
Apo E - Control				Apo E - 1% Chol.			Apo E - 3 % Chol.		
Tissue	B max (fmol/mg)	Kd (nmol)	n	B max (fmol/mg)	Kd (nmol)	n	B max (fmol/mg)	Kd (nmol)	n
Heart	1590 ± 390	0.92 ± 0.4	5	1260 ± 370	1.57 ± 0.8	7	2 580 ± 1890	1.62 ± 0.9	7
Aorta	24 500 ± 4100	1.9 ± 1.0	9	16 670 ± 3800 **	1.48 ± 0.5	7	20 800 ± 6850	2.68 ± 1.44	7

**Table 4.** Average  $B_{\max}$  values **fmoles / mg** protein and  $K_d$  values (nM) of [ $^3$ H]PK 11195 binding to TSPO in aorta and heart homogenates of WT (Bb-Control) and apoE KO mice, fed with standard feed, and feed supplemented with 1% and 3% cholesterol (Chol). One-way analysis of variance ANOVA was used, with Mann-Whitney as the post-hoc, non-parametric test. Data are expressed as mean ± SD; \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$  vs. control.

### 3. Discussion

There is strong evidence that accumulation of plasma derived lipoproteins in the arterial wall launches specific cell reactions that account for atherosclerosis process: enhanced NO production, amplification of the inflammatory response, apoptosis, endothelial function impairment, enhanced smooth muscle cell migration and proliferation, and macrophage foam cell formation (Steinberg et al., 2002; Whitman, 2004; Zhao et al., 2008; Singh et al., 2009). Mice lacking apoE have a substantial delay in the metabolism of lipoproteins, particularly VLDL, even fed with a regular standard chow feed (Hoen et al., 2003; Kato et al., 2009). Lesions in apoE-deficient mouse have many features in common with human atherosclerosis, even that the progression can be advantageous in many experimental situations (Dansky et al, 1999). At 26 weeks, atherosclerotic lesions are in the early stages of development, characterized by lipoprotein accumulation, leukocyte gathering, and foam cell formation. This model develops atherosclerotic lesions which progress to occlusion of

coronary artery by 8<sup>th</sup> to 11 months after regular feeding (Piedrahita et al., 1992; Whitman, 2004). Aged (42-54 weeks) apoE KO mice develop intraplaque hemorrhage and plaque instability features, accelerated by feeding westernized diets (Seo et al., 2005; Singh et al., 2009). We found, similar to previous observations, advanced fibrous plaque development accompanying prolonged cholesterol feeding (Figure 1C) in apoE mice but not in WT mice. Another study by Molnar et al. (2005) showed that although high fat feeding induced endothelial cell dysfunction in WT mice, it did not enhance neointimal formation in WT mice. Also in WT rats, a high fat, high cholesterol diet does not appear to lead to atherosclerosis, although modest morphological alterations in the aortic wall could be observed (Dimitrova-Shumkovska et al., 2010a).

We also checked in blood plasma of apoE KO and WT mice the levels of total cholesterol, including triglycerides, high-density lipoprotein and low-density lipoprotein, since it can increase the risk of heart disease and atherosclerosis (Steinberg, 2002; Stocker and Keany, 2004, 2005). Mice naturally have high levels of HDL and low levels of LDL, lacking the cholesterol ester transfer protein, an enzyme responsible for trafficking cholesterol from HDL to VLDL and LDL. As reported also by others previously, we found clear cut differences in abundance of cholesterol related particles between apoE KO mice and WT mice (Table 1), (Hoen et al., 2003; Kato et al., 2009). In particular, each group of apoE KO mice had five times more plasma cholesterol than their WT counterparts. The apoE KO mice also always had higher TAG levels. HDL levels in apoE KO mice supplied with standard feed and 1% cholesterol supplemented diet was also twice as high than in WT mice. Interestingly, 3% cholesterol supplemented diet resulted in a reversal, meaning that HDL levels (i.e. "good" HDL-lipoproteins) in WT mice became twice as high as in apoE KO mice (Table 1). The generally low LDL cholesterol levels in WT mice even with cholesterol supplemented diet may be due to the capability of WT mice to efficiently suppress the percentage of dietary cholesterol absorption by increasing the excretion of gallbladder biliary cholesterol concentration (Sehayek et al., 2000).

We used this model, of apoE KO mice fed with cholesterol supplemented diet that shows well developed atherosclerosis, to assess oxidative stress in the aorta in correlation with TSPO binding density and atherosclerosis. For this purpose, homogenates of the aorta were used for ROS analysis and antioxidant enzymes activities. As accumulation of proatherogenic lipid affects all cell types present within the vascular wall, the response of the entire tissue vs. isolated cells to the hyperlipidemic conditions is relevant as an indication of vascular defense as a whole. The increase in plasma cholesterol levels was paralleled by changes in oxidative stress parameters in WT mice and ApoE KO mice, as discussed in detail below.

An indicator of cellular defence capacity against oxidative stress is the presence of reduced GSH, which we determined in the aorta homogenates after application of feed with cholesterol supplements. As seen in table 3, a reduction of GSH content in was evident compared to the corresponding controls, when 3% cholesterol diet was administered to WT as well as apoE mice. This shows that cholesterol diet regime indeed constitutes an elevated risk factor for ROS formation, due to a reduction in GSH levels in this model. It has been

reported that ROS induce vascular cells to express cell adhesion molecules that trigger adhesion of leukocytes to the endothelium, which is part of the initiation atherosclerosis (Yang et al., 2009). Interestingly, it was also found that TSPO expression correlates positively with expression of adhesion molecules (Bode et al., 2012; Veenman et al., 2012). This may suggest that the reduction in TSPO levels seen in this study may counteract adhesion of leukocytes to the endothelium, and thereby prevent initiation atherosclerosis in particular in WT mice.

In accord with the observations of Hoen et al. (2003) that the mRNA levels of many antioxidant enzymes in apoE KO mice are higher (1.5 -5 fold) in the age of 6-15 weeks, compared to aged-matched wild type mice, we also saw that SOD activity were higher in aorta homogenates of apoE mice than those in age-matched WT mice (Table 3). Their hypothesis is that the aorta compensates for the oxidative stress induced by atherogenic stimuli, by stimulating the expression of antioxidant enzymes, thereby delaying the process of atheroma plaque formation. The latter was supported by Yang et al. (2004, 2009) providing evidence that over expression of catalase and superoxide dismutase delayed the development of atherosclerosis in apoE KO mice.

To determine the potential involvement of the TSPO in effects of apoE dysregulation, we studied TSPO binding density in heart and aorta of apoE KO mice (B6.129P2-apoE<sup>tm1</sup> N11) versus their wild type (WT) background mice, with and without inclusion of 1% and 3% cholesterol to the diet. TSPO has been detected in heart of normal mice before, and we found comparable levels in our control animals (Hashimoto et al., 1989; Weizman et al., 1992; Fares et al., 1990; Katz et al., 1994; Dumont et al., 1999). To our knowledge the present study is the first study regarding TSPO binding density in the aorta of mice, which are quite high (even comparable to TSPO levels in adrenal of rats (Gavish and Fares, 1985; Gavish et al., 1999)). We found that enhanced cholesterol levels in the diet can result in reduced TSPO binding density in the aorta and heart of WT mice, as well as in the aorta of apoE mice (Table 4). The present study indicates that there is negative correlation between ROS parameters in heart tissue and TSPO binding density in cholesterol fed WT mice. Namely, in the heart of WT mice, the “steady state” levels of lipid peroxides (TBARs) showed a 2.5 fold enhancement after 3% cholesterol supplemented diet vs. a 1/3 fold enhancement in the group with 1% cholesterol supplemented diet. Regarding oxidized proteins in the heart tissues of WT mice fed with cholesterol supplements, AOPP and proteins carbonyls showed increases of 40% and 35%, respectively, regardless of the cholesterol percentage (data not shown). Such a relation between ROS parameters and TSPO binding density is not apparent in apoE mice, since in apoE mice little effect is seen on TSPO binding density.

Also in a previous study, enhanced plasma lipid levels due to HFHC diet supplied to rats, enhanced oxidative stress parameters and decreased indicators for antioxidant activity in the aorta, which were associated with reduced TSPO density in this organ (Dimitrova-Shumkovska et al., 2010a). Notably, wild type rats are not prone to develop atherosclerosis even when subjected to HFHC diet (Dimitrova-Shumkovska et al., 2010a). We have shown that reduction of TSPO expression by genetic manipulation in vitro in cell culture reduces mitochondrial ROS generation (Veenman et al., 2008, 2012; Zeno et al., 2009). We have

discussed previously that the reduced TSPO levels accompanying atherogenic challenges may be a compensatory mechanism to counteract oxidative stress in the aorta and liver (Dimitrova-Shumkovska et al., 2010 a, b, c). This would be in effect similar to increased levels of SOD observed, which also counteract oxidative stress (see above). Our present study suggests that reduced TSPO binding density as observed in WT mice subjected to cholesterol supplemented diet may counteract oxidative stress as one mechanism to attenuate the development of atherosclerosis. As TSPO binding density is not affected in apoE mice subjected to cholesterol supplemented diet mentioned TSPO dependent mechanism is not available for apoE KO mice to counteract development of atherosclerosis. Presently, it is not known which components of the vascular wall, i.e. mast cells, smooth muscular, or dermal vascular endothelial cells, would be important for the potential correlation between TSPO expression, oxidative stress, and atherosclerosis (Stoebner et al., 1999; 2001; Morgan et al., 2004; Veenman and Gavish, 2006; Dimitrova-Shumkovska et al., 2010 a, b, c).

It can be assumed from the present study, that oxidative stress parameters do not absolutely correlate with the development of atherosclerotic lesions (because supplementation with 1% of cholesterol to the diet does not affect oxidative stress), but the absolute levels of cholesterol do correlate with atherosclerotic development. Nonetheless, enhancement of cholesterol percentage from 1% to 3% in the diet resulted in significant increases in ROS parameters of WT and apoE KO mice in comparison to their control groups, and also provoked advanced lesion formation in aortic intimae in apoE KO mice fed a 3% cholesterol supplemented diet (but not in WT mice). TSPO binding density is reduced due to cholesterol intake in particular in WT mice and such changes in TSPO binding density in WT mice are in negative correlation with oxidative stress measured in heart and aorta. We believe the reductions in TSPO binding density in WT mice are compensatory for oxidative stress and atherosclerotic development. Thus, the lack of a significant decrease in TSPO binding density in the aorta of 3% cholesterol fed apoE KO mice may actually correlate with the enhanced atherosclerosis in this model. The capability of apoE KO mice fed with 1% cholesterol to reduce TSPO binding density in the aorta may present a rudimentary anti-atherosclerosis protective capacity. In conclusion, this study is in accord with previous studies suggesting that reductions in arterial TSPO binding density are part of a mechanism counteracting the development of atherosclerosis. A question is how the presence of apoE, in combination with enhanced dietary cholesterol levels, can result in suppression of TSPO binding density. It is also important to find out how in a mechanistic sense a reduction in TSPO levels can contribute to self protection against the development of atherosclerosis.

**Explanation of abbreviations and symbols:** ACS, acute coronary syndrome; ANOVA, analysis of variance; (AOPPs), advanced oxidation protein products; ApoE<sup>-/-</sup> KO, apolipoprotein E knockout mice; cAMP, adenosine 3,5-cyclic monophosphate; CBR, central-type benzodiazepine receptor; DBI, Diazepam Binding Inhibitor; CAM, cell adhesion molecule; CVD, cardiovascular disease; HDL, high-density lipoprotein; HFHC- high fat high cholesterol diet; HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A reductase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; Hb, hemoglobin; IL-1, interleukin-1 (IL-2, etc.); kDa, kilodalton; Kd,



equilibrium dissociation constant;  $K_m$ , equilibrium constant related to Michaelis-Menten kinetics (similarly,  $K_d$ ,  $K_a$ ,  $K_{eq}$ ,  $K_s$ ); LDL, low density lipoproteins; mPTP, mitochondrial permeability transition pore; MCP-1, monocyte chemoattractant proteins-1; NADP, nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; PBR, peripheral-type benzodiazepine receptor; PC protein carbonyls; PK 11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide; ONOO<sup>-</sup>, peroxynitrite; Ro5-4864, (4'-chlorodiazepam); ROS, reactive oxygen species; SOD, superoxide dismutase activity; TBARS, thiobarbituric acid reactive substances; TNF, tumor necrosis factor; TSPO, 18 kDa translocator protein; VCAM-1, vascular cell adhesion molecule; VSMCs, vascular smooth muscle cells.

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