UNIVERSITA' DEGLI STUDI DEL MOLISE

Department of Medicine and Health Science "Vincenzo Tiberio"

PhD School in Translational and Clinical Medicine

XXX Cycle - S.S.D.: MED/50

Role of Splenic Sympathetic Nervous System / Placental Growth Factor (PlGF) axis in obesity-induced arterial hypertension.

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Academic Year: 2016/2017

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1. Introduction.

1.1   Definition and Epidemiology of obesity.

It is widely accepted from both researchers and clinicians to the world health organization (WHO) that obesity can be described as an excessive and aberrant accumulation of adipose tissue. The easier and most powerful strategy to establish overweight or obesity is the BMI (Body Mass Index) calculated as: person's weight (kg) divided by the height's (m) square. Generally, individuals with an equal or higher BMI value of 25 kg/m^2 are considered overweight; whereas when BMI is equal or higher than 30 kg/m^2, persons are considered obese (**Ortega FB et al. Circulation Research 2016; World health organization. Obesity: preventing and managing the global epidemic. Report of a WHO Consultation WHO Technical Report Series 2000)**. Global distribution of the prevalence of overweight and obesity is reported in **Figures 1** and **Figures 2**.

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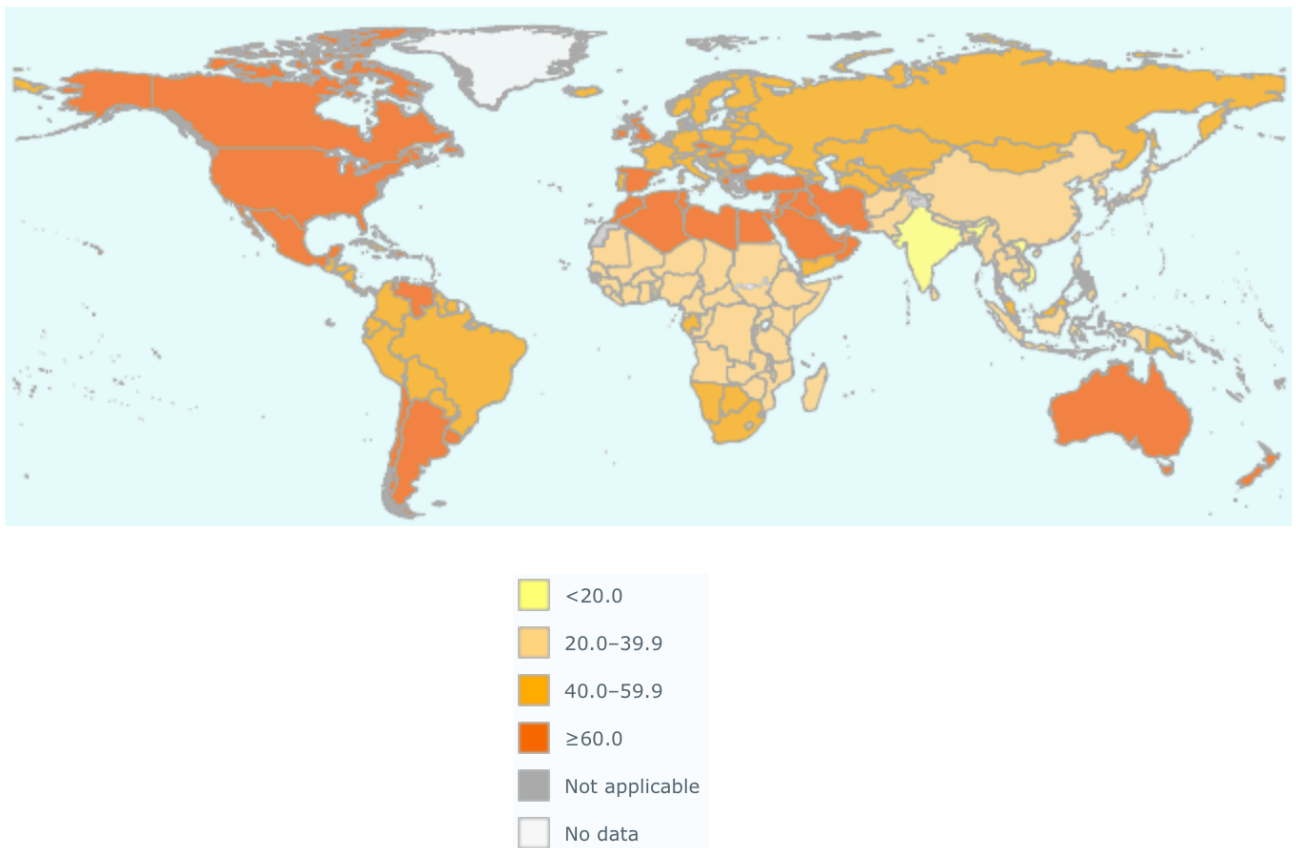
Figure 1: Graphical representation of overweight prevalence among male and female adult global population (http://www.who.int/en/).

Obesity, and other metabolic disease as Diabetes Mellitus (DM), has gained even more attention as a relevant and increasing public health burden, swiftly reaching global epidemic proportion, that embrace not only the economic sphere but also the social one. A recent study revealed disquieting evidences regarding the prevalence of obesity in the adult and young population (**Ng M et al. Lancet 2014**).

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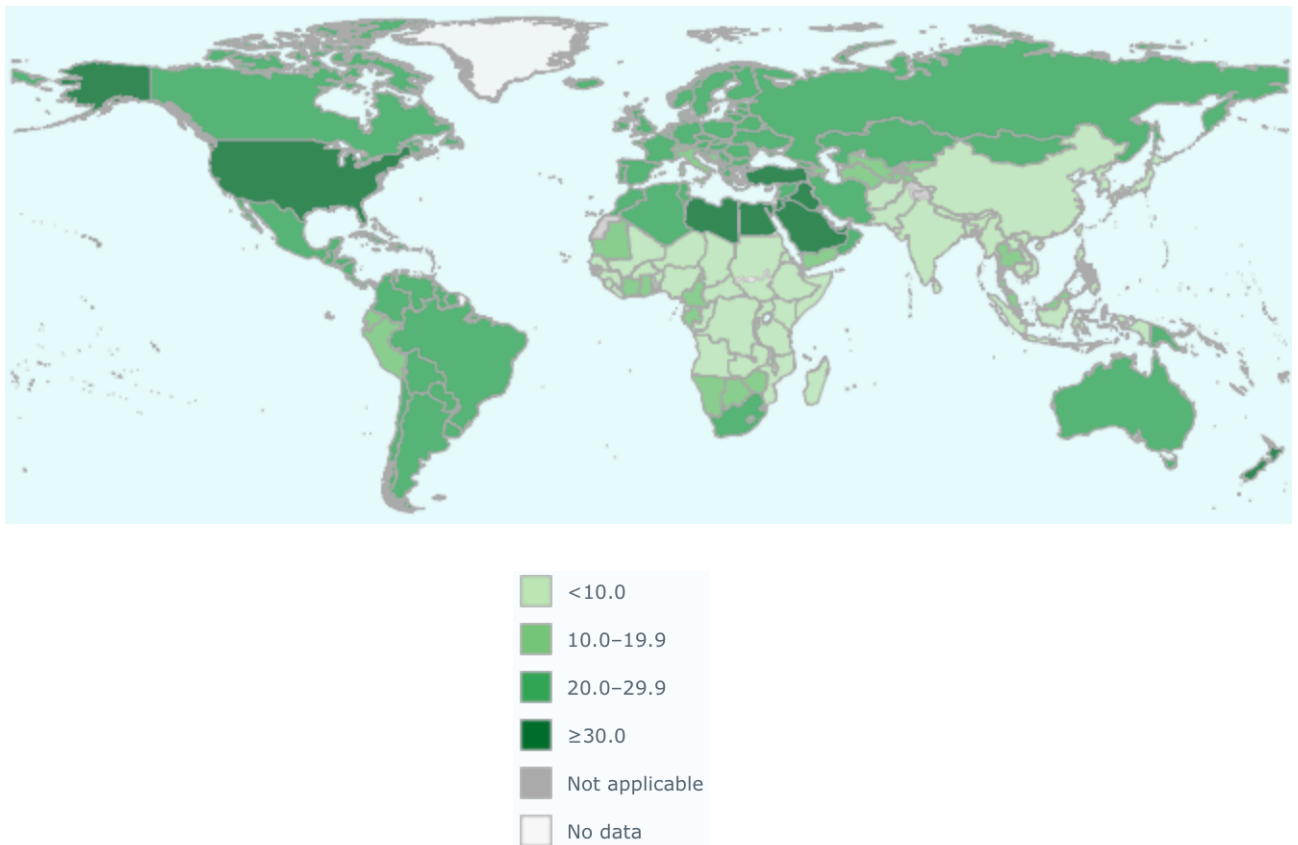
Figure 2: Graphic represents global prevalence of obesity among male and female adults (http://www.who.int/en/).

Data collected showed that the prevalence of obesity has increased dramatically since 1980 involving both men and women (**Ng M et al. Lancet 2014, Flegal KM et al. JAMA 2016**). Despite in developed country such increasing trend seems to have reached the peak and evolved toward a plateau, in developing country obesity prevalence is still rising. Higher rates of obesity and overweight have been observed in the United States, Middle-East, Central America, Pacific and Caribbean Islands with proportions that in some cases broadly exceed the 50% for men or women. In

Western Europe as well as in Canada the prevalence of obesity reaches about 20% (**Ng M et al. Lancet 2014**).

The most dramatic evidence concern data regarding children and adolescents. In the last three decades, the incidence of overweight and obesity among younger population has dramatically increased (**Bhupathiraju SN et al. Circ Res 2016, Ogden CL et al. JAMA 2016**). Singer and Lumeng have recently reviewed the critical stage of obesity development during childhood emphasizing the note that obesity is a lifespan, chronic disease which have repercussions in adulthood health (**Singer K et al. J Clin Invest rev 2017**). The most recent data, describe that in the last 20 years the prevalence of obesity in young people has continued to rise. The more children become heavier, the more is the possibility to develop cardiovascular complications (**Baker JL et al. N Engl J Med 2007**) and it has been demonstrated that development of obesity in childhood has important repercussion in adulthood with earlier onset of cardiovascular disease (**Agirbasli et al. Card Ther 2016**). For these reasons, it is imperative the need of deep and massive efforts to counteract such global issue.

Overall the main causes of the uncontrolled increase of obesity prevalence in both developed and developing country, could be ascribed to the progressive change in human habits towards a more sedentary lifestyle associated with higher caloric food consumption, contributed to the spreading of this systemic and crippling disorder (**DeMarco VG et al Nat rev endocrinol 2014**). Nevertheless, obesity is an entangled disease that cannot be ascribed solely to derangements in caloric intake and energy expenditure but different causal aspects, spanning from genetic to environmental ones, occur to determine this complex disease.

1.2   Obesity and Metainflammation.

The term "metabolic syndrome" summarizes a variety of different disease in which obesity is a central feature and responsible for the increasing risk for other pathologies development as insulin resistance and type 2 diabetes, respiratory diseases as Asthma, some type of cancer, neurodegenerative disease as dementia and importantly, cardiovascular diseases including atherosclerosis, stroke and hypertension.

In general, fat pad is the tissue designated to store energy that is released in response to metabolic need. Evolution, in fact, has positively selected mechanisms that allow accumulation of energetic molecules to deal with critical condition, as starvation or limited food availability, to preserve homeostasis (**Hotamisligil GS et al. Nature 2017**). On the other hand, however, no system or molecular pathway has been positively selected to face energetic overabundance, and its uncontrolled intake brings irreversibly to a pathological environment.
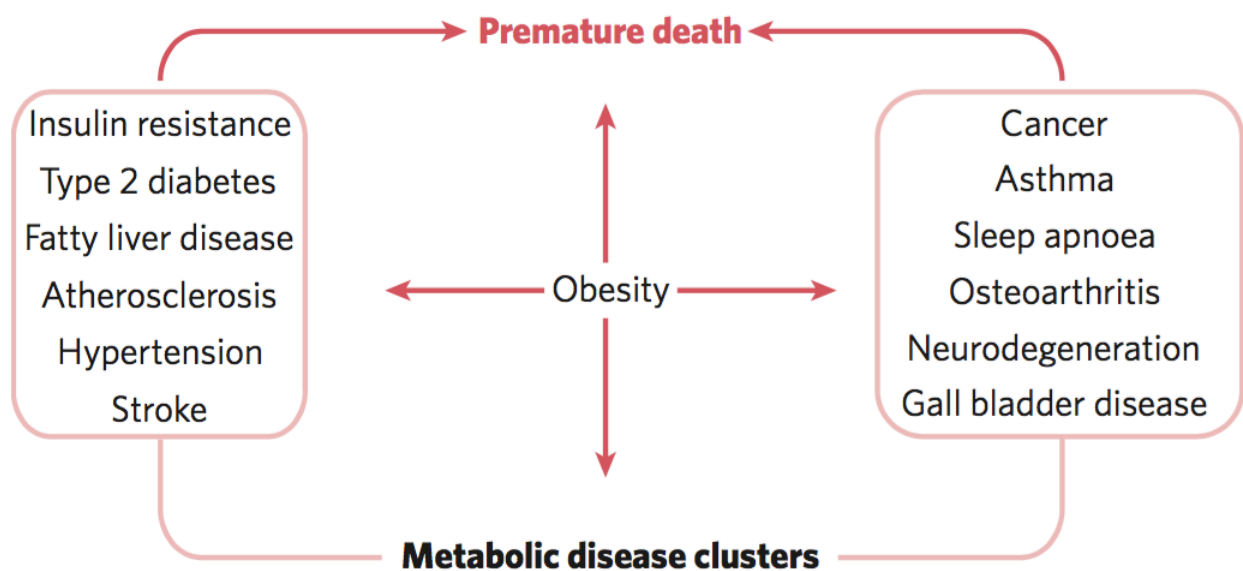
Figure 3: Obesity is a pivotal risk factor for the development of a wide range of metabolic associated diseases. Schematic overview of metabolic disease cluster associated with obesity (**Hotamisligil GS et al. Nature 2017**).

Increase in size and structural alteration along with switch of cellular properties are critical features that characterize adipose tissue in the initial stages of obesity development (**Lumeng CN Diabetes 2008**).

Immune system assumes a critical and indispensable role presiding responses to a wide variety of stimuli that have to be activated to ensure a fast, effective and incisive reaction to protect host from enemy injury but, at the same time, that need to be turned off to prevent deleterious long-lasting immune reaction.

Classically, in order to counteract noxious challenges, immune system evokes a concerted and well-tuned response to restore a physiological environment. As a result, when the harmful agent has been eradicated, that vigorous but short-term immune activation returns to basal level, re-establishing the correct immune homeostasis. Differently, when the immune response is not able to overwhelm the damaging injury, the prolonged and not resolved immune activation could lead to worse outcome. In this view, metabolic disease, present features of inflammation that are completely different from the classic inflammatory paradigm lacking the presence of "dolor, rubor, calor and tumor". The "chronic" but "low grade" inflammation, has profound consequences in the critical derangement of metabolic homeostasis (**Hotamisligil GS Nature 2006**; **Lumeng CN J Clin Invest 2011**). Such pathological condition has been renamed as "meta-flammation" defining the metabolically triggered inflammation.

Importantly, prolonged and unresolved inflammation in metabolic tissue as fat pad, muscle and brain, is a key component that depict metabolic disorders especially obesity, type 2 diabetes and cardiovascular disease. The interest in unveiling the way in which metabolism affects immune system and conversely how immune system is involved in metabolic pathway regulation gave rise to a new field of investigation: immunometabolism (**Cildir G et al. Trends Mol Med 2013)**.

Globally, fat pad can be divided in three categories: brown, beige and white that differ in body distribution, metabolic activities and function; each of these in turn can be subdivided in different subcategories. Among white adipose tissue (WAT),

increased visceral adipose tissue (VAT) has been identified to be a risk factor for insulin resistance and cardiovascular disease (**Cildir G et al. Trends Mol Med 2013, Lim S et al. Int J Cardiol 2013**).

Adipose tissue contains not only adipocyte, but also harbors pre-adipocytes, endothelial cells and, importantly, immune cells. Additionally, adipose tissue releases in the blood stream important molecules as chemokines, adipokines and chemokines that participate in maintaining homeostatic environment or promote pathological development through an endocrine or paracrine functions.

Both resident and infiltrating immune cells in the adipose tissue are conditioned by metabolic alteration and, in turn, can stimulate the worsening of metabolic derangements. For that reason, dysfunction in adipose tissue caused by infiltrating immune cells, is a critical aspect of hypertension or other cardiovascular disease.

In 1993, the association between a component of the immune system (Tumor necrosis factor-alpha, TNF-α) and obesity-induced insulin resistance was demonstrated for the first time in a mouse model of obesity (**Hotamisligis GS Science 1993**), with increased proportion of TNF-α production during obesity development. Some years later, in a study conducted in humans, it was demonstrated that adipose tissue of obese individuals produce more TNF-α, and this occurrence was associated with insulin resistance (**Hotamisligil GS J Clin Invest 1996**) and increased BMI (**McLaughlin T et al. J Clin Invest 2017**).

In a short time, it has become even more clear the pivotal role that immune system assumes in the fine regulation of metabolic homeostasis.

During the onset of obesity, fat pad tissue undergoes profound alterations involving its structural and functional properties along with, and even more significantly, resident and recruited immune cell phenotype.

In healthy physiological condition, adipose tissue immune cells are all focused in maintaining the balanced environment. M2 macrophages and T lymphocyte regulatory cells, known as Treg cells, promote the anti-inflammatory environment with primary production of interleukin-10 (IL-10) and transforming growth factor-

beta (TGF-β), or other as IL-4, IL-5, IL-13 from Th2 aiming at constraining tissue inflammation (**Winer S et al. Nat Medicine 2009, Lumeng CN et al. Diabetes 2008, Adamson SE et al J Clin Invest 2016**). Fine regulation and production of anti-inflammatory mediators to maintain stable homeostasis is not ascribed only to macrophages or Treg cells but also to a proportion of IL-10 producing innate-like B cells and Adipose natural regulatory B cells (Breg) that populate visceral adipose tissue of healthy individuals (**Nishimura S et al. Cell Metab 2013, Wu L et al. PNAS 2014**).

On the other side, increased adipose tissue and development of obesity promote the recruitment of pro-inflammatory M1 macrophages overwhelming protective M2 macrophages and organizing in characteristic crown like structures that surround necrotic adipocytes (**Lumeng CN et al. JCI 2007, Cildir G et al. Trends Mol Med 2013, Apovian CM et al. ATVB 2008, Kranendonk ME et al. Atherosclerosis 2015, Zheng C et al Cell Death Dis 2016**). Pro-inflammatory macrophages sensing environmental pro-inflammatory cytokines as IFN-γ and in turn are stimulated to produce pro-inflammatory mediators as TNF-α, IL-1β, IL-6 and nitric oxide (NO) (**Winer DA et al. Nat Med 2011**). The sustained accumulation of macrophages, and consequential production of inflammatory molecules, promote the recruitment of other pro-inflammatory cells reinforcing inflammation and metabolic disease development.

Proinflammatory CD8+ and CD4+ T cells along with Natural killer (NK) cells produce IFN-γ participating to increase pathological inflammatory environment (**Winer S et al. Nat Medicine 2009, Wensveen FM Nat Immunol 2015, Lee BC et al. Cell Metab 2016)**.

Moreover, evidences in DIO mouse models and human study highlighted the role played by Dendritic cells in presenting antigen peptide to T cells promoting their differentiation (**Cildir G et al. Trends Mol Med 2013**) and finally it has been reported in murine models that Neutrophils are recruited in the earliest phase of high-fat diet feeding (**Elgazar-Carmon V et al. J Lipid Res 2008**).

It is even more clear that both vascular and metabolic dysfunction with immune derangements are pivotal aspect that altogether occur in vascular diseases (**Guzik TJ Cardiovas Res 2017**)

## 1.3   Cardiovascular diseases: a focus on hypertension.

Important analyses defined blood pressure elevation, overweight and obesity, high cholesterol levels as some of the most critical causes of cardiovascular insults (**Ezzati M et al. Lancet 2003**). Cardiovascular disease are the most diffused chronic diseases that affect human population all over the world with higher prevalence among developing countries than the already developed ones. It has been estimated that one third of deaths are related to cardiovascular dysfunctions and high blood pressure is responsible in large proportion.

Even more evidences correlate blood pressure with the risk of incidence with a variety of comorbidities including kidney- and cardiovascular disease as stroke or hypertension. Hypertension is defined as a chronic increase in blood pressure, promoting end-organ damage and resulting in increased mortality. The latest categorization refers that normal blood pressure levels should be lower than 120 mmHg for systolic blood pressure (SBP) and 80 mmHg for diastolic blood pressure (DBP). Hypertension is defined with SBP higher than 130 mmHg and higher than 80 mmHg for DBP (**Whelton PK et al. Hypertension 2017**).

Great efforts have been spent to investigate the pathophysiological mechanisms underlying the pathogenesis of hypertension.

The causes of blood pressure increase, in the most part of cases, remain obscure. The term "secondary hypertension" refers to hypertension for which the causes have been established. For the other cases, in which no causes are known, that are the main percentage, are referred to "essential hypertension". Arterial blood pressure is

subjected to continuous fluctuation and is constantly modulated by multiple factor (**Figure 4**): gene variants and interactions, the environment and at physiological level, neuronal and hormonal factors contribute to the control of blood volume, cardiac output and vascular resistance (**Cowley Nat Rev Genet 2006**).
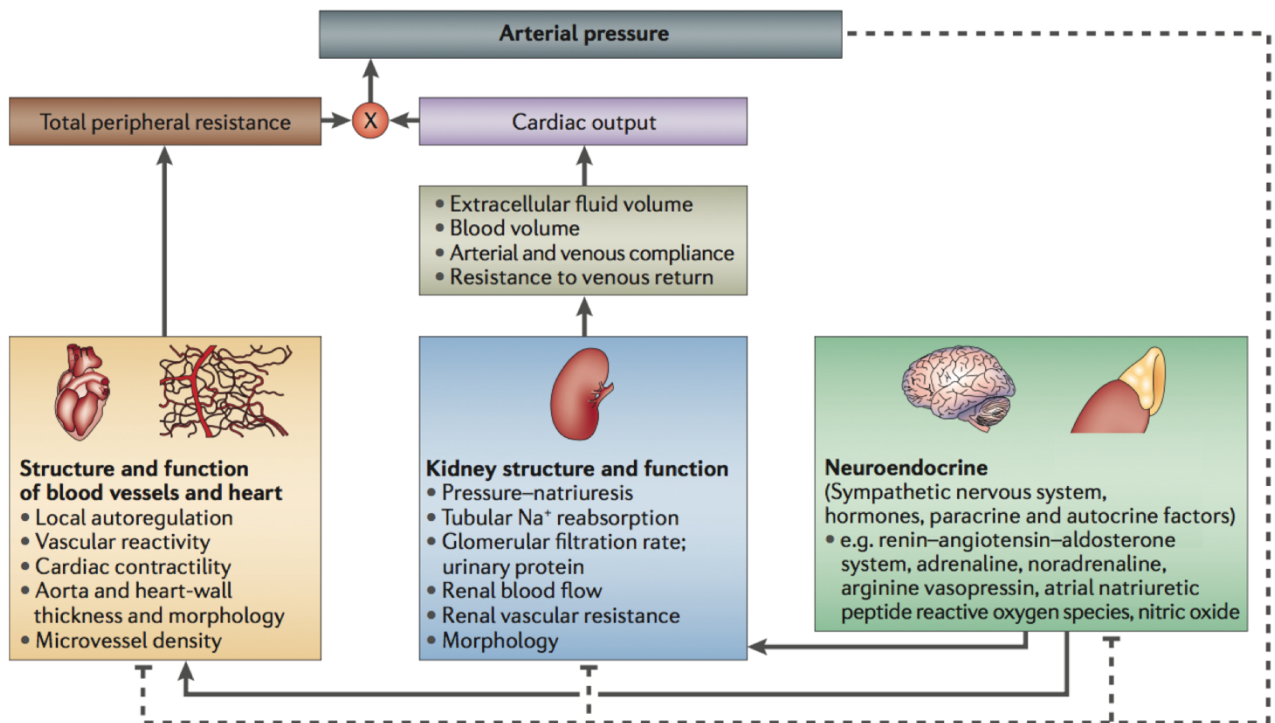
Figure 4: Schematic summary of arterial blood pressure regulation pathway. (Arrows represent function exerted, Dotted lines represent negative feedback control) (**Cowley AW et al. Nat Rev Genet 2006**)

Renin-angiotensin–aldosterone system (RAAS) is probably one of the most essential blood pressure control system involving endocrine components. Since its pivotal role, already little derangements related to this pathway could lead to the development of hypertension. Firstly, inactive pro-renin is produced by kidney and released in the blood stream where is converted into renin, its active form (**Danser AH et al. Nephrol Dial Transplant 2007**). Now renin is responsible for the converting reaction from

Angiotensinogen to the inactive Angiotensin I (Ang I). At this point, Ang I can be converted by the Angiotensin–converting enzyme (ACE) in Angiotensin II (Ang II) (**Figure 5**). Ang II is the principal active molecule in RAAS pathway promoting important rise in blood pressure since its vasoconstriction effect and being involved in short-term but also long-term modulation of blood pressure (**Munoz-Durango N et al. Int J Mol Sci 2016; Hall JE et al. Compr Physiol 2012**). The more Ang II is produced, the more renal excretory activity is impaired with pressure natriuresis derangements.

Angiotensin-(1-7) is an active product that derives from the cleavage of Ang I by specific endopeptidases or the cleavage of Ang II by angiotensin-converting-enzyme 2 (ACE-2). Angiotensin (1-7) gained important attention since its protective and opposite effect from Ang II on cardiovascular system (**Santos RA Hypertension 2014**).

Aldosterone is another important effector involved in the RAAS pathway. Production and release of Aldosterone from the adrenal gland is stimulated by Ang II increase. Effects of this hormone are directed to modulate blood pressure mediating sodium and water retention and potassium excretion (Spat A and Hunyady L Physiol Rev 2004). Aldosterone, indeed, binds mineral corticoid receptors, activating transcription of genes involved in electrolyte and fluid homeostasis (**Munoz-Durango N et al. Int J Mol Sci 2016**).

Thus, it is clear the pivotal role exerted by RAAS system in blood pressure control. It is even more evident that such system needs to be finely balanced since expression impairments in any of these molecules could lead to damaging consequences including hypertension and alteration of organ structure and function.

Different therapeutic approaches have been developed to reduce RAAS activity targeting Ang II receptor, ACE, or mineralcorticoid-receptor (MR) promoting a correct sodium homeostasis aiming at reducing blood pressure level (**Hall JE et al. Compr Physiol 2012**). Alternative therapeutic strategies could involve Angiotensin-(1-7) enhancing its protective effects (**Santor RA Hypertension 2014**).
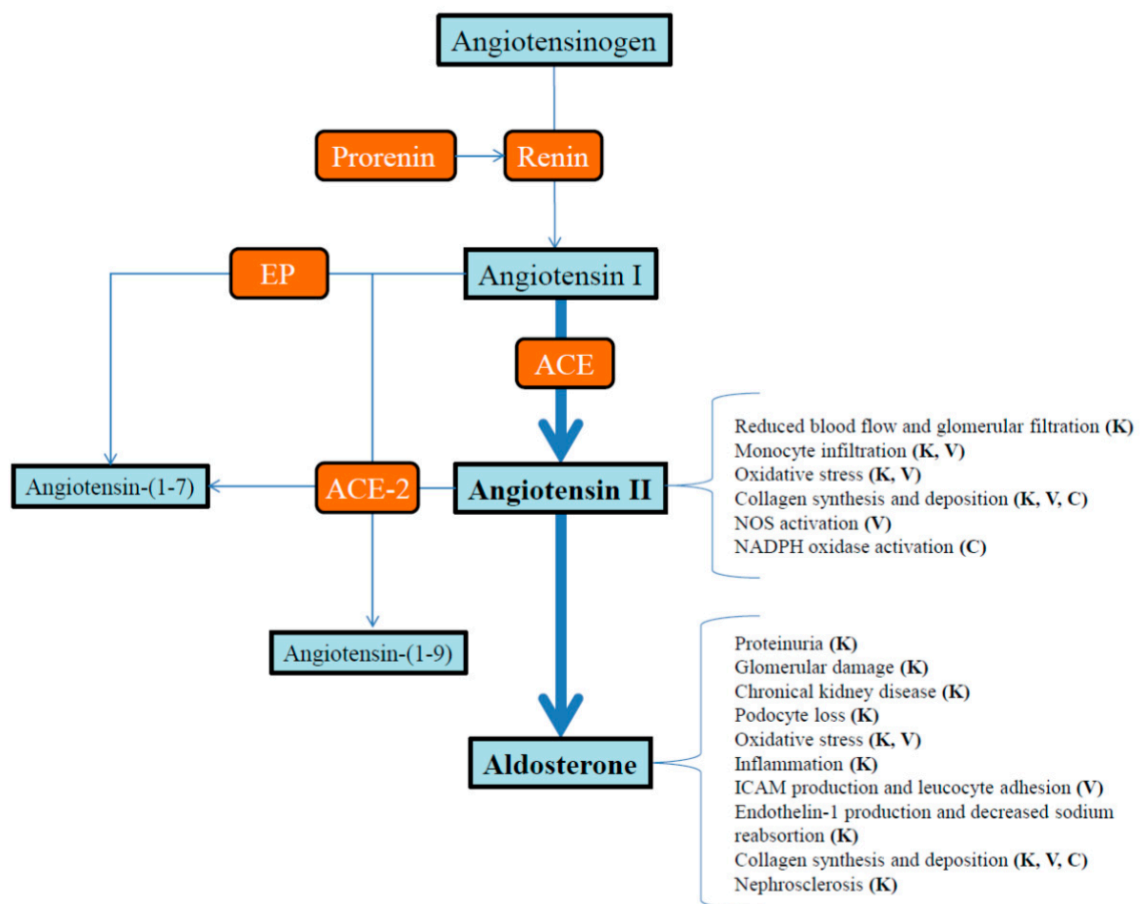
Figure 5: Schematic description of the renin-angiotensin-aldosterone-system pathway (**Munoz-Durango N et al. Int J Mol Sci 2016**).

1.4   Obesity, Cardiovascular disease and Sympathetic nerve activity.

An epidemiological study focused on obesity, thoroughly reported the strong relationship with increased risk of heart failure or hypertension (**Prospective study collaboration Lancet 2009**), being an independent risk factor for cardiovascular disease affecting independently both men and women. Among obese patients, the prevalence of hypertension exceeds 60%. The incidence of hypertension is positively related to BMI and even just a little increase could be responsible of higher risk to develop hypertension. Long-term prospective study has highlighted that individuals

developing weight gain or obesity during early phase of mature age and middle-age were subjected to higher risk for the onset of hypertension. Overall, the study focuses on the fact that maintaining a normal controlled weight confers a lower risk to develop cardiovascular disease (**Shihab HM et al. Circulation 2012**). In this way, clinical trials have shown that reducing body weight through improved eating habits or physical activity, is a compelling behavior to prevent hypertension or reduce blood pressure level in hypertensive patients (**Neter JE et al. Hypertension 2003**).

The abnormal pathophysiological changes that endure adipose tissue in obesity, promoting an aberrant expression of a wide range of inflammatory mediators, firstly affect systemic metabolism and particularly contribute to the development of cardiovascular diseases. It is also clear that different fat depots have dissimilar consequences on the possibility to develop cardiovascular disease as confirmed by numerous studies conducted on humans and animal models, attributing to visceral adipose tissue a critical role in insulin resistance and cardiovascular disease development (**Fuster jj et al. Circulation research 2016, Sironi AM et al. Hypertension 2004)**.

Adipose tissue not only harbors immune cells that secrete pro- or anti-inflammatory immune modulators, but it also produces and releases proteins called adipokines (**Fuster JJ et al. Circ res. 2016, Lori A. et al. IJMS 2017**) that are involved in the regulation of multiple functions including appetite, energy expenditure, glucose metabolism, blood pressure and inflammation. The majority of Adipokines, such as Leptin, TNF-α, IL-6 and resistin and others, are characterized by pro-inflammatory activity (**Fuster JJ et al. Circ res. 2016, Ouchi N et al. Nat rev immunol 2011, Nakamura K et al J Cardiol 2014**). On the contrary, adipose tissue produce a smaller quantity of anti-inflammatory adipokines as Adiponectin or Secreted frizzled-related protein 5 (Sfrp5) (**Fuster JJ et al. Circ res. 2016, Ouchi N et al. Science 2010**). Some of them, play their role reaching the Central Nervous System, influencing neuron activity in distinct brain areas. This is the case of Leptin. Leptin is a hormone highly and specifically expressed by adipose tissue. Its production is positively related to the

amount of fat pad, thus circulating levels are highly increased during obesity, developing, as a consequence, leptin resistance (**Maffei M et al. PNAS 1995**). The principal action of this adipokine is related to the control of energy balance influencing appetite: increased level of leptin suppresses appetite and enhances energy expenditure resulting in weight loss (**Rahmouni K et al. Hypertension 2007**). Crossing the Blood Brain Barrier, Leptin reaches the Central Nervous System exerting its function on feeding behavior and body weight (**Zlokovic BV et al. Endocrinology 2000, Malpas SC et al. Physiol rev 2010**). The regulation of this function is addressed in the mediobasal hypothalamic arcuate nucleus to the melanocortin system. Here two neuronal population: the orexigenic agouti-related peptide (AgRP) and neuropeptide (NPY) neurons and the anorexigenic proopiomelanocortin (POMC) neurons, modulate feeding behavior and energy expenditure in response to metabolic stimuli, with opposite function (**Jais A et al. JCI 2017, Belgardt BF et al. Ann N Y Acad Sci 2010)**.

Besides this function, leptin has also pro-inflammatory action and its aberrant overproduction promotes cardiovascular disease development (**Fuster JJ et al. Circ res. 2016**). Despite during obesity the effects of leptin on energy balance is lost caused by leptin resistance, its ability in activating sympathetic nerve activity is preserved with detrimental consequences for the cardiovascular system increasing arterial pressure (**Harlan SM et al. Circ res 2011, Rahmouni K et al Diabetes 2005, Malpas SC Physiol rev 2010**). Such notion is corroborated by the evidence that acute infusion of Leptin in obese MICE induced renal sympathetic nerve activity increase but, conversely, the Leptin anorexigenic property was blunted (**Mark AL. et al. J. Hypertension 2002**).

A critical factor that is involved in blood pressure rise during obesity is the sympathetic nervous system overdrive and many interesting works have revealed the close relationship between weight increase and sympathetic nerves activation (**Mark AL et al. Hypertension 2009, Hall JE Circ res 2015**). Sympathetic nerves reach important organs as kidneys modulating its functions. Significantly, increased

sympathetic discharge to these organs has been related to the onset of hypertension (**Rahmouni et al. Hypertension 2014**). It has been widely demonstrated, with different animal model of obesity (i.e. diet-induced obesity on rabbits (**Lim K et al. Hypertension 2016**), mice (**Chhabra H et al. Mol Metab 2017**) or rats (**Muntzel MS et al. Hypertension 2012**), the involvement of Leptin in the over-activation of sympathetic tone (**Grassi G et al. Circ Res 2015**). Moreover, researchers highlighted the fact that obesity enhances sympathetic activity that plays a crucial role in hypertensive disease development (**Rahmouni et al. hypertension 2014, Carnevale et al Nat Commun 2016, Simonds SE et al. Cell 2014**).

Evidences from both diet induced obesity mouse models and human studies show that the melanocortin system is involved also in blood pressure regulation during obesity (**Rahmouni K et al. Hypertension 2007, Purkayastha et al Nat Med 2011**); abrogating Leptin-POMC neuron signaling pathway, in fact, protects against obesity-induced hypertension (**Do Carmo et al. Hypertension 2011**). The action of Leptin on renal sympathetic nerve activity in the central nervous system is not restricted to the arcuate nucleus, rather different research groups have characterized similar effects also in the brain subfornical organ (**Young CN et al. Hypertension 2013**) demonstrating that different brain areas modulate leptin metabolic and cardiovascular effects.

1.5   Placental growth factor: much more than an angiogenic factor.

Placental growth factor (PlGF) was firstly determined and cloned in 1991, from human placenta library of cDNA (**Maglione D et al. Proc. Natl. Acad. Sci. 1991**) and belongs to the family of the Vascular Endothelial Growth Factors (VEGFs). Besides PlGF, other structurally related members fall into the same family as VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E (**Dewerchin M et al. Cold Spring Harb Perspect Med 2012)**. Similarly to all VEGF family members, human PlGF proteins exist in different

isoforms (PlGF-1, PlGF-2, PlGF-3 and PlGF-4) due to alternative splicing processes. Differently, only the isoform PlGF 2 is encoded by murine *PlGF* gene (**De Falco S et al. Exp Mol Med 2012**). The importance of this family members is related to the main function of vasculogenesis and angiogenesis during pregnancy, tissue growth, wound healing but are also critically involved in the development of important diseases as cancer or amyotrophic lateral sclerosis. All members exert their activity assembling as dimer proteins but PlGF, along with VAGF-A and VEGF-B, can also generate heterodimeric structures (**De Falco S et al Trends Cardiovasc Med 2002**).

VEGF-A (or VEGF) was the first discovered member of VEGF family exerting its primary function in vascular growth. VEGF-A is an essential gene since its experimental deletion or blockade was lethal already at embryonic developmental stage (**Carmeliet P et al. Nature 1996, Ferrara N et al. Nat Med 2003**) promoting angiogenesis derangements. Its importance has been clarified also subsequently to the embryonic phase at neonatal development stage through severe kidney glomerular impairments (**Ferrara N et al. Nat Med 2003**). On the other side, VEGF antibodies administration in nude mice was effective in inhibiting progression of different hematological malignancies (**Ferrara N et al. Nat Med 2003; Goel HL and Mercurio AM Nat Cancer rev 2013**). Thus, the relevance of VEGF actions at different stages of individual development suggests that its expression must be regulated in spatial and temporal manner.

VEGF-A, VEGF-B and PlGF, exert their biological function binding specific type of related tyrosine kinase receptor: VEGF receptor-1 (VEGFR-1 also named as Flt1) and VEGF receptor-2 (VEGFR-2 or Flk1) (**Autiero M et al. J Thromb Haemost 2003**) (**Figure 6**). The dimeric form of VEGF-A binds VEGFR-1 and VEGFR-2 but the homodimeric form of PlGF and VEGF-B are able to bind only VEGFR-1 receptor (**De Falco S et al. Exp Mol Med 2012**). Finally, co-receptors, Neuropilin-1 and Neuropilin-2, have been described as important mediators of VEGF-A and PlGF receptor interaction (**De Falco S et al. Trends Cardiovasc Med 2002; Ferrara N et al. Nat Med 2003**) (**Figure 1**).

VEGFR-1 and Neuropilin-1 have been identified also as soluble receptors as a consequence of mRNA alternative splicing. The presence of soluble receptor is of fundamental importance for the spatial and temporal regulation of VEGF-A and PlGF, generating gradients necessary for the correct development of the organism, modulating their locally as well as systemically action.
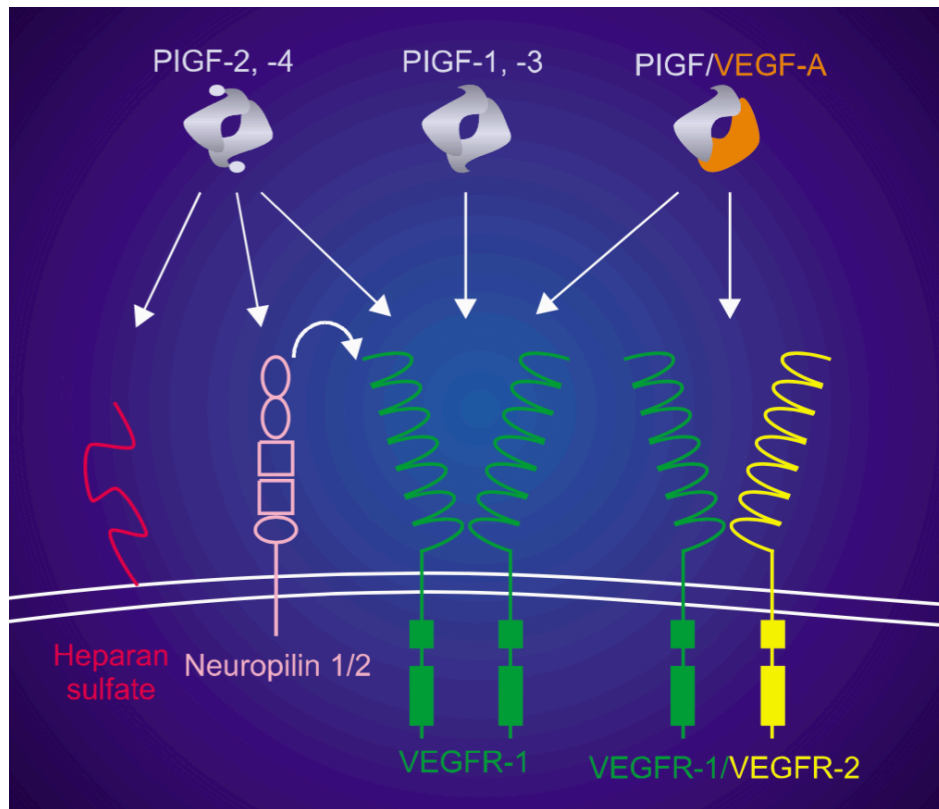
Figure 6: Schematic model of PlGF isoforms homodimers and PlGF-VEGF heterodimer binding affinity with Neuropilin 1 and 2, VEGFR-1 and VEGFR-1/VEGF-R2 receptors (**De Falco S et al. Exp Mol Med 2012**).

Continuous insights in PlGF function, highlighted the fact that PlGF could play a role in VEGFR-2 activation through different modalities. First of all, Carmeliet et al. observed that the affinity of PlGF for VEGFR-1 could displace VEGF-A allowing its interaction with VEGFR-2 (**Carmeliet P. et al. Nat Med 2001**). In second instance, in studies

related to tumor development and angiogenesis, PlGF and VEGF-A could co-operate forming heterodimer. In such conformation, PlGF-VEGF interacts with both VEGFR-1 and the dimer VEGFR-1/VEGFR-2 (**Tarallo V et al. Cancer res 2010; Autiero M et al. Nat Med 2003**). Lastly, VEGFR-2 could be activated through transphosphorylation in response to PlGF-VEGFR-1 interaction with the consequence of further enhancing VEGF-induced angiogenesis (**Autiero M et al. Nat Med 2003**).

Since its discovery, PlGF was found expressed in several different cells and even more works concurred to increase knowledge about its functions. The first purpose in which PlGF was described was related to angiogenesis. Ziche et al., taking advantage of both "*in vivo*" and "*in vitro*" esperiments, demonstrated for the first time that PlGF was able to induce angiogenesis and moreover to enhance endothelial cells migration and proliferation (**Ziche M et al. Lab Invest 1997**). Some years later, genetic manipulation allowed to generate PlGF-deficient mice and it was observed that these mice were healthy and fertile with a normal vascular development (**Carmeliet P et al. Nat Med 2001**). Thus, since PlGF is expressed at early stage during embryonic development, its deficiency overall does not affect systemic development.

Other functions in which PlGF was found being involved concern the stimulation of mesenchymal fibroblast proliferation and migration; the derangemnts in dendritic cell differentiation along with antigen recognition; it is involved in neuronal devolment promoting generation of Schwann cells and play important role in cancer growth (**Dwerchin M and Carmeliet P Cold Spring Harb Perspect Med 2012**). A schematic representation of PlGF expression and involvement in different aspect of cellular functions (**Figure 7**).
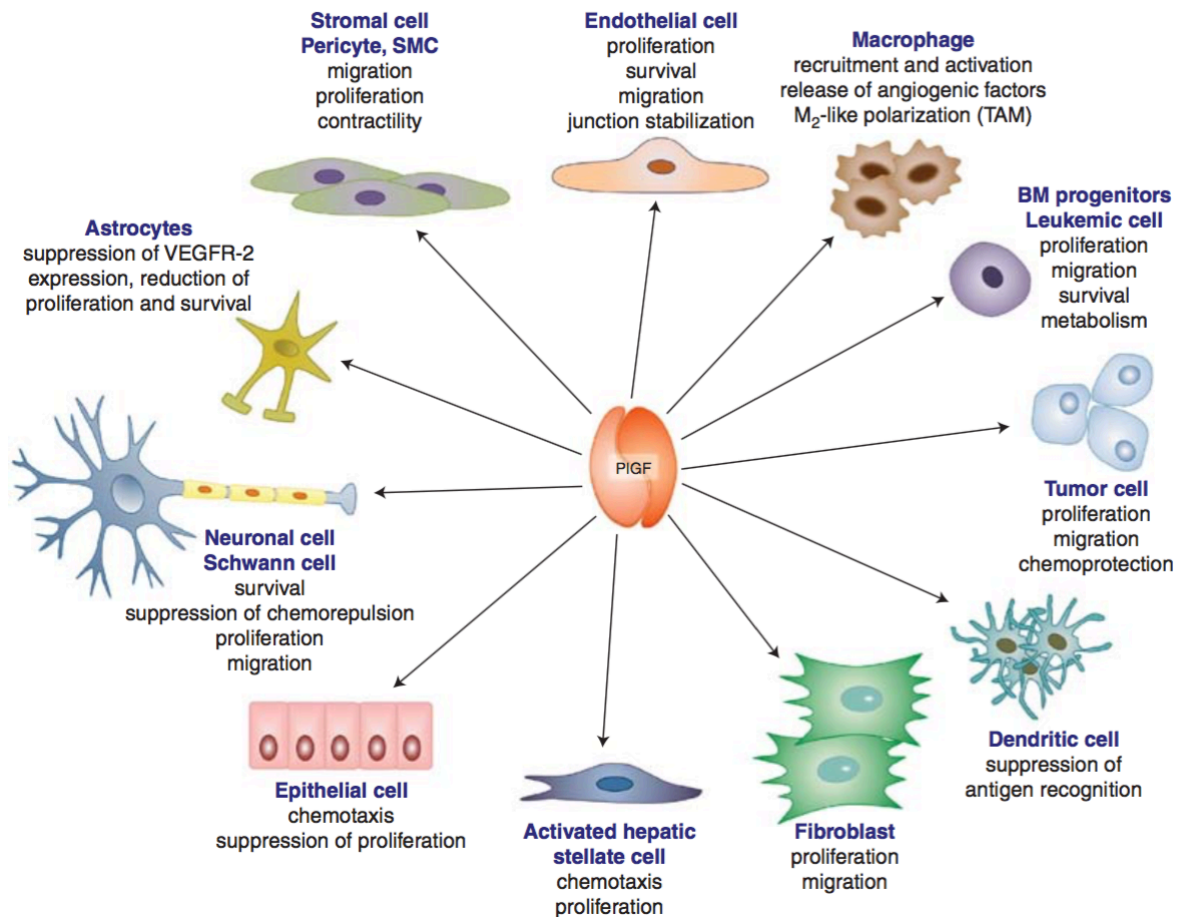
Figure 7: Schematic overview of PlGF pleiotropic action ranging from proliferative regulation to cellular migration and activation affecting both vascular and non-vascular cells (**Dwerchin M and Carmeliet P Cold Spring Harb Perspect Med 2012**).

In addition to these properties, PlGF has been also characterized as an endothelial cells and monocytes chemotactic factor, acquiring the property of "angiogenic cytokine" modulating both angiogenesis and inflammatory response during cardiovascular disease (**Carnevale D and Lembo G Trends Cardiovasc Med 2012**). In light of this, evidence from mouse model of cardiac remodeling induced by Toracic Aortic Coarctation (TAC) revealed that PlGF expression is increased and again PlGF is pivotal in modulating inflammatory activation providing the correct inflammatory environment to allow adaptive cardiac remodeling (**Carnevale D et al Circulation 2011**). Conversely, PlGF deficiency caused inflammatory derangements with aberrant

cardiac compensatory hypertrophy incurring irretrievably in heart failure (**Carnevale D et al Circulation 2011**).

Such evidence highlighted the importance of a fine balancing of immune response not only on metabolic syndrome but also in cardiovascular disease and PlGF gained even more importance, beyond its angiogenic properties, for a pivotal immune regulator.

1.6   Neuro-immune modulation of hypertension.

A great amount of studies unraveled the role carried out by immune cells for the onset of hypertension (**Harrison DG J Am Soc Hypertens 2013, Madhur MS and Harrison DG Circ Res 2012, Guzik TJ et al. J. Exp. Med. 2007**). Mouse model of hypertension induced by Angiotensin II infusion was used to decipher the role exerted by T cells. Mice lacking both T and B cells were used by Guzik et al. to demonstrated that T cells infiltration in target organs of hypertension as the aortic adventitia are essential to promote blood pressure rise (**Guzik TJ et al. J. Exp. Med. 2007**). More recently, valuable experiments based on chimeric mice with splenic transplantation clarified, for the first time, that renal and vascular infiltrating T lymphocytes induced by chronic Angiotensin II infusion, were of splenic origin (**Carnevale et al. Immunity 2014**). Otherwise, It was demonstrated from different works that Deoxycorticosterone acetate (DOCA)-salt stimulate the Renin Angiotensin system activating Angiotensin II receptors in the brain Subfornical organ (**Hilzendeger et al Hypertension 2013**) and that the same hypertensive stimulus is able to enhance sympathetic drive in the spleen (**Carnevale et al. Nat Comm 2016**). Conversely, the consequences on splenic immune activation evoked by different known hypertensive stimuli, such as high-fat diet, has not been fully clarified.

The interplay between immune- and nervous-system was further demonstrated with other evidence suggesting a role for the cholinergic signaling pathway in mediating immune response, showing that galantamine, an acetylcholinesterase inhibitor, was able to hamper the expression of both Tumor necrosis factor (TNF) and IL-6 with anti-inflammatory effects in endotoxemic mice (**Pavlov VA et al. Brain Behav Immun. 2009**) and, moreover, lowered inflammation, body weight, Insulin resistance along with other aspects related to metabolic syndrome in mouse model of high-fat diet induced obesity (**Satapathy et al. Mol Med 2011**). These aspects of neuro-immune interaction in metabolic syndrome deserve deepen study to unravel possible implication with cardiovascular diseases.

A possible link between metabolic syndrome, neuro-immune response and hypertension could be achieved by the Placental growth factor (PlGF).

First evidence of the importance of PlGF in modulating immune response in mouse model of cardiovascular disease were described by Carnevale et al. in 2011 studying PlGF in cardiac response to transverse aortic constricton (TAC). Since the peculiar features of PlGF, as on one hand an angiogenic factor and on the other acts as a cytokine, was demonstrated that it is responsible for correct and fundamental inflammatory response after TAC (**Carnevale et al. Circulation 2011**). PlGF-deficient mice, in fact, showed lower monocytes activation and inflammatory cytokine expression and consequential marked worsening of both structural and functional cardiac parameters (**Carnevale et al. Circulation 2011**).

In 2014, Carnevale et al. clearly described for the first time a new role for the splenic factor PlGF in modulating Sympathetic nervous system and immune cells in response to a hypertensive stimulus as chronic infusion of Angiotensin II. PlGF-deficiency protected mice from the development of hypertension. The importance of the spleen and the splenic PlGF is highlighted by experiments in which splenectomy or selective ablation of sympathetic innervation to the spleen through celiac ganglionectomy protected mice from blood pressure rise as well, realizing that the splenic PlGF is pivotal for hypertensive disease development (**Carnevale et al. Immunity 2014**).

PlGF was found highly expressed along the marginal zone of the spleen in response to Angiotensin II infusion and its activation is necessary for T cell stimulation and egression from the splenic reservoir toward the classical target organs of hypertension: kidneys and aorta (**Carnevale et al. Immunity 2014**).

1.7 The Spleen: anatomical and functional properties for neuro-immune interaction.

Anatomically, the spleen is located into the abdomen, under the diaphragm and in contact with the stomach. Despite initially was depicted as a simple blood filter, the spleen has gained even more attention among the scientific community since it exerts a previously unrecognized role in immune system modulation. The spleen is encircled by a capsule made up of elastic and fibrous tissue. Starting from the capsule derive the trabecule, constituted by connective tissue, directed to the parenchyma of the spleen and that encompass nerves lymphs and blood vessels. The capsule together with trabeculae sustain the spleen conferring solidity to the structure and an organized environment (**Cesta MF Toxicol Pathol 2006; Mebius RE and Kraal G Nat Rev Immunol 2005; Lori A et al. Int J Mol Sci 2017**). Central arterioles originating from splenic artery spread through the trabeculae structure toward the splenic White Pulp (WP) (**Lori A et al. Int J Mol Sci 2017**). The WP is configured as a lymphoid structure in which both T-cells and B-cells are confined in specific areas near arterial vessels in response to chemoattractant molecules (**Mebius RE and Kraal G Nat Rev Immunol 2005**). Splenic marginal zone (MZ) is a critical area that is placed at the interface between WP and blood circulation. Along the MZ reside different population of immune cells that play active role in the surveillance against blood circulating antigens (**Magri G et al. Nature immunology 2014**). Blood that flows through the MZ reach the Red Pulp (RP) and venous sinuses where important function of blood filtration is exerted by specific macrophages subtracting old

erythrocytes, promoting reaction against bacterial infiltration and eventually iron recycling. Hereafter, blood flow through trabecular area directed toward the splenic vein (**Lori A et al. Int J Mol Sci 2017**) (**Figure 8**).

Another relevant immune activity that occurs in the RP is the production of antibodies. Plasmablast, firstly differentiate within the WP in response to specific antigen. Plasmablast overexpress CXC-chemokine receptor 4 that specifically binds the CXC-chemokine ligand 12 (CXCL12) highly produced in the RP and involved in mmigration and maintenance of cells in this specific zone of the spleen. In parallel, the repression of CXCR5 and CCR7 receptors on plasmablast along with the reduced response to the WP homing chemokine signals (**Mebius RE et al Nat Rev Immunol 2005; Hargreaves et al. J exp Med 2001**).

The splenic lymphoid area, residing in the WP, need a strictly and finely regulated system to preserve its cellular distribution. CXCR5 specific B-cell receptor, is fundamental for the B-cell follicles arrangement allowing B cell differentiation.

Likewise, CCL19 and CCL21 CC-chemokines ligands force both T-cells and Dendritic Cells (DCs), that specifically express CCR7 receptor on their membrane surface, into the WP T-cell zone (**Lori A et al. Int J Mol Sci 2017**). Derangements in the pattern expression involving these chemokine or related receptor, could result in aberrant immune cells localization from their appropriate splenic place (**Mori S et al. J exp med 2001; Ramos-Perez WD et al Nat immunol 2015**).

In the end, MZ is the connecting structure placed between circulating blood in the RP and the immune reservoir in the WP. Such configuration provides an efficient system to protect from blood-derived pathogens through relevant production of immunoglobulins (IgA, IgM, IgG) from B-cells producing B-cells (**Puga I et al. Nat Immunol 2012**). In addition to their action as Ig producers, B-cell could also act as an antigen presenting cell (APC) able to activate CD4+ T cells (**Rubtsov AV et al. J Immunol 2015**).

Overall, besides B-cells, splenic MZ is populated by a wide variety of immune cells: Neutrophils, Dendritic cells and Macrophages conferring a complete and effective immunosurveillance (**Puga I et al. Nat Immunol 2012; Gatto D et al. Nat Immunol 2013; Ravishankar B et al. Proc Natl. Acad. Sci. U S A. 2014**)

Since the last two decades, researchers made several steps forward in unveiling the basis of nervous-immune system communication. Neural mechanisms are pivotal regulators of several biological systems including the inflammatory reflex (**Rosas-Ballina M Tracey KJ Neuron 2009; Carnevale D et al. Immunity 2014; Carnevale D et al. 2016 Nat comm 2016; Cole SW et al. Nature rev cancer 2015**). Great efforts have been focused on the characterization of the splenic innervation; indeed, it has been described that fibers of the sympathetic nervous system innervate the spleen and that their synapses are in closely proximity with immune cells (**Carnevale D et al. Nat comm 2016; Rosas-Ballina M et al. Science 2011; Andersson U Tracey KJ J Exp Med 2012; Lori A et al. Int J Mol Sci 2017**) (**Figure 8**).

Neurotransmitters from the sympathetic nerves bind specific receptor on immune cell surface, generating a "synapse-like contact", and influencing their activity in promoting or decreasing cytokine expression (**Carnevale D et al. Immunity 2014; Straub RH et al. Trends Pharmacol Sci 2004; Lori A et al. Int J Mol Sci 2017**). Evidences showed that immune cells, in turn, can influence Noradrenaline (NA) release from sympathetic synapses and also in that case enhancing or reducing its production. Immune cells release of adrenocorticotrophic hormone (ACTH) on one hand and IL-6, IL-2 or TNF on the other induce opposite consequence on NA production acting on pre-terminal autoreceptors on the neuronal cell surface (**Straub RH et al. Trends Pharmacol Sci 2004**). For these reasons, the neuro-immune interplay consists of a highly intricate but extremely well-tuned regulating processes.
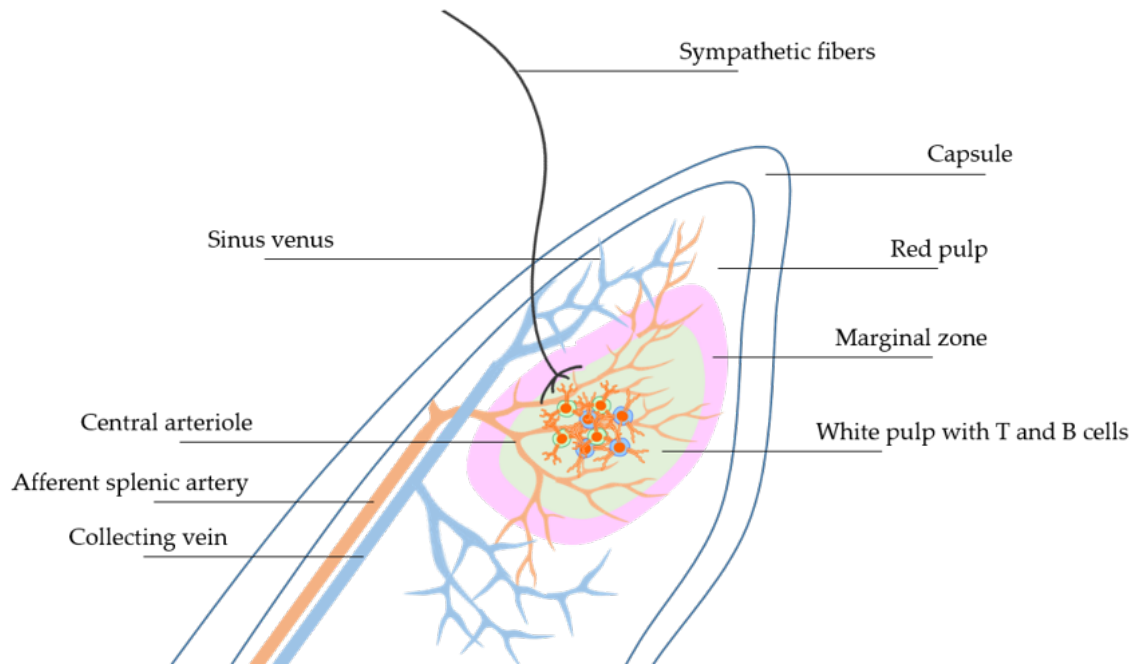
Figure 8: Schematic description of the architectural organization of splenic areas containing various immune cells: White Pulp (WP) and Red Pulp (RP) with Sympathetic nerve fibers innervating Marginal Zones (MZ) **(Lori A et al. Int J Mol Sci 2017).**

Overall, all these evidences support the fact that the spleen is an important lymphoid organ that hosts both component of the innate and adaptive immune system providing an essential role in immunosurveillance. Moreover, the neuro-immune interaction between sympathetic fibers and the splenic immune cells is of key relevance to understand novel mechanisms in the pathogenesis of hypertension **(Carnevale D et al Immunity 2014; Carnevale D et al. Nat Comm 2016**).

1.8 Animal model of diet-induced obesity.

In order to understand the pathophysiological aspect of human diseases, developing animal models are an unavoidable need. Moreover, the importance to dissect disease-associated complications and the possibility to deepen the molecular pathway that manage the pathological onset or progression of the disease are of critical relevance. Progressive and continuous developments enabled to critically discover biological features of a wide range of disease. The first goal, would be to be able to translate such new insight from the bench to the bedside for new and more powerful therapeutic treatment.

The significance to generate animal model for studying disease also resides in the opportunity to produce *in vivo* studies and deepen the complexity of many aspects in an entire biological system. Animal models, indeed, are chosen because they share extensive similarity with humans concerning physiological, anatomical and genetic aspects.

Rodents, and in particular mice and rats, are probably the most attractive animal model to study for metabolic and cardiovascular disease. The possibility to modify genome sequences through single nucleotide modification, or even the insertion or deletion of a DNA section, originating protein with loss of function, gain of function or completely abrogate their production, gave rise to the generation of genetic mutant rodent models of pivotal importance for several different studies and therapeutic approaches with the most advanced discoveries in molecular medicine.

Concerning metabolic disease and cardiovascular disease, mice and rats are probably the most appropriate, and studied, animal models.

A large amount of studies regarding metabolic syndrome focus on obesity being a central feature of this multifactorial disorder. Genetically modified mice, such as Leptin deficient mice (ob/ob mice) or mouse that carry a mutation in the leptin receptor gene (db/db mice) have been widely used to dissect obesity and its related comorbidities (**Giesbertz et al. Diabetologia 2015**; **Drel VR et al. Diabetes 2006**). Nevertheless, such genetic mutation, have been discovered only in a little part of

human population (**O'Rahilly Nature 2009; Varga O. et al. Obesity Rev 2009; Simonds et al. Cell 2014**). Thus, another model, that probably is more representative of the incidence in human population without involving genetic proceedings, is represented by diet-induced obesity. In general, most of the mice or rats fed with a high-fat diet progressively accumulate body mass becoming obese. However, it has been evidenced that different strains could manifest different responsiveness in term of weight gain or metabolic related dysfunction as glucose tolerance or insulin resistance (**Rossmeisl et al. Diabetes 2003; Montgomery et al Diabetologia 2013**). For example, C57BL/6J or AKR/J strains, despite different response in insulin resistance and glucose tolerance (**Rossmeisl et al. Diabetes 2003**) demonstrate more susceptibility to high-fat diet induced obesity. On the other hand, BALB/c background strain show great protection against glucose metabolism derangements induced by obesity (**Montgomery et al Diabetologia 2013**). Research based on these mouse model, and many others, have contributed and will provide even more insight to deepen metabolic disease, obesity and related cardiovascular complications (**Calligaris SD et al Plos One 2013; Simonds SE et al Cell 2014; Asirvatham-Jeyaraj N et al Hypertension 2016; Weisbrod RM et al Hypertension 2013**).

2. Aims of the study.

The aims of this study were focused on the following points:

1. To develop a murine model of diet-induced obesity.

2. To explore the relevance of Splenic Sympathetic Nervous System in the onset of obesity-induced hypertension.

3. To explore the involvement of Placentar Growth Factor in obesity development and onset of obesity-induced hypertension.

3. Methods.

3.1   Mice:

All the experimental procedures were performed according to European Communities guidelines and the Italian legislation on animal experimentation. The adopted protocol was approved by the Italian Ministry of Health and by our Institutional Committee.

Wild-type C57BL/6J mice were purchased from Charles River and used for sympathetic nerve recording, splenectomy and celiac ganglionectomy.

PlGF-deficient mice, from the original strain in 50% 129Sv/50% Swiss, were backcrossed for 11 generations in C57BL/6J mice as previously described (Carnevale et al. 2014, Gigante et al. 2006). As control, were used PlGF WT mice with the same genetic background (C57Bl/6J).

3.2   Diet-induced obesity paradigm:

At 6 weeks of age, all mice started the diet protocol for 16 weeks. Mice with free access to water, were fed a low-fat diet (LFD, 10% kcal fat; catalog #D12450J; Research Diets) or a high-fat diet (HFD, 60% kcal fat; catalog #D12492; Research Diets) *ad libitum* for 16 weeks (**Figure 9**).

PlGF WT and knockout mice weight gain and diet intake were monitored weekly.
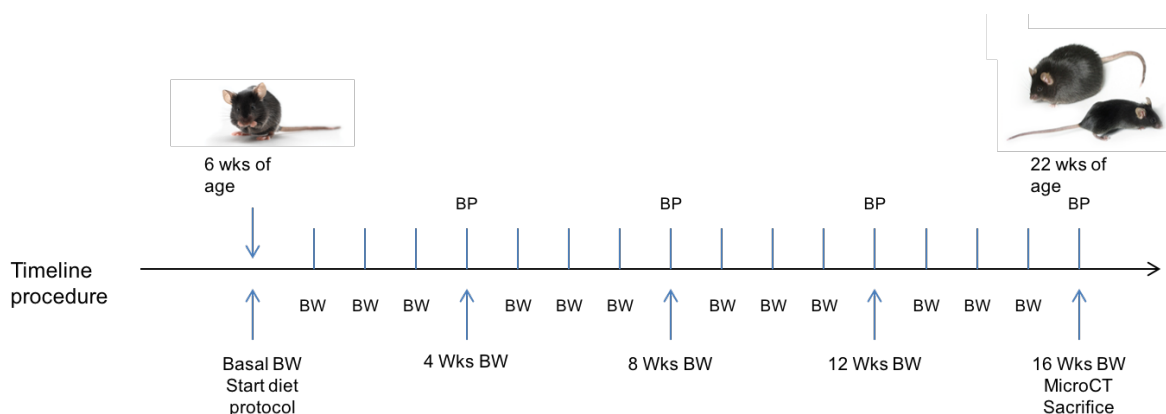
Figure 9: Schematic overview of diet-induced obesity diet protocol. BW: body weight measurements, BP: blood pressure measurements.

3.3   Electrophysiological recording:

Electrophysiological recording was performed as previously described (**Carnevale D et al. Nat. Comm. 2016**). Mice were anaesthetized with 5% isoflurane and maintained with 1.5–2% (supplemented with 1 l min^{-1} oxygen). Blood pressure was continuously monitored with a single-pressure catheter (Millar, SPR-100) implanted in the left femoral artery and connected to a pressure transducer (Millar, MPVS ULTRA). Body temperature was monitored and maintained between 37 and 38 °C. After an abdominal incision, the intestinal tract was carefully moved exposing the splenic artery that was isolated, and the splenic nerve separated from tissue. A bipolar stainless-steel electrode (MLA1214 Spring Clip Electrodes, ADInstruments) was positioned on the splenic nerve. The ground wire was plugged into mouse soft tissues. Splenic SNA was recorded from the implanted electrode continuously for the next 2 h. Mice were then killed by isofluorane overdose, and Splenic SNA was recorded for a further 30 minutes to estimate post-mortem residual activity and background noise. The splenic nervous signal was amplified (gain 10,000) and successively sampled at 4 kHz with a digital amplifier (Animal Bio Amp; ADInstruments). The raw signal was filtered at 300–1,000 Hz and expressed as mV. Data were collected using a Power Lab data acquisition system and analyzed with Lab Chart 7 (Spike Analysis Module). The sympathetic burst was identified as a signal above the threshold determined by the background noise of the post-mortem recording. Firing frequency was calculated as the mean value of total number of spikes in a time window. Whereas the amplitude gain was evaluated as the mean spike amplitude and spike identification threshold ratio in the time window.

3.4   Blood pressure measurements:

Arterial blood pressure was measured by tail cuff system as previously described (Carnevale et al. 2014). Tail cuff plethysmography (BP-2000 Series II, Visitech Systems) was used to assess blood pressure between 9 am and 1 pm. Blood pressure

was measured for 5 consecutive days every 4 weeks since the beginning of diet procedure.

3.5   Celiac Ganglionectomy:

Wild-type C57BL/6J mice at the age of 5 weeks, were anesthetized with Isoflurane (2-5 Vol %), supplemented with 1L/min oxygen. The abdominal cavity of mice was opened through a midline laparotomy, after Aorta and celiac artery isolation, the celiac ganglion was exposed and excised. As controls, sham wild-type C57BL/6J mice were equally anesthetized and celiac ganglion exposed but not removed.

3.6   Splenectomy:

Wild-type C57BL/6J mice at the age of 5 weeks, were maintained under isoflurane anesthesia (2-5 Vol%), supplemented with 1L/min oxygen. Abdominal cavity was opened and after accurate splenic vessel cauterization, the spleen was exposed and carefully removed. As control, was followed the same procedure in C57BL/6J mice at the same age, the spleen was exposed but not removed.

3.7   MicroCT:

MicroCT scanning was performed with SKYSCAN 1178 (SKYSCAN, Kontich, Belgium), as previously described (**Carnevale D et al. Immunity 2014**). The tube parameters were set to 50 kV and 615 µA, exposure 480 ms, with a rotation step of 0.360°. Mice were anaesthetized with ketamine-xylazine and positioned on the bed. After image reconstruction the analysis was performed as follows: a ROI was placed from the top of the ribcage to the tail of the animal, the fat was segmented by thresholding in the range of [-300 ;-50] HU (**Metzinger MN et al. Sensors (Basel) 2014**), the global volume was segmented with an automated thresholding (Otsu) to discriminate background and foreground of an image. The numerical analyses were performed

using an in-house software based on OpenCV, the visual reconstruction was performed by Slicer3D (**Fedorov A. et al. Imaging 2012**).

3.8   <u>Immunofluorescence analysis:</u>

Immunofluorescence analysis was performed as previously described (**Carnevale D et al. Immunity 2014**).

Briefly, the spleen was collected and successively embedded in OCT. 25μm sections from spleen were post- fixed with 4% paraformaldehyde for 15 min and processed for staining with the following primary antibodies:

- Rat anti-CD169 (1:200; Serotec) specific for metallophilic marginal zone macrophages;

- Rat anti-ER-TR7 (1:200; Acris) specific for fibroblast reticular cells;

- Hamster anti-CD3 (1:100; Serotec), specific for T cells labeling;

- rat anti-CD45R/B220 (1:50; BD Pharmigen) specific for splenic B cells labeling;

- Rabbit anti-PlGF (1:250; Abcam) specific for PlGF labeling.

Sections were incubated with the appropriate secondary antibodies conjugated to Alexa Fluor 488 or Cy3. Thereafter, sections were coverslipped with DAPI-containing mounting medium (Vector laboratories) and then evaluated with Zeiss 780 confocal laser-scanning microscope. A 405 Diode laser was used to excite DAPI; a 488 nm argon laser to excite Alexa Fluor 488 and a 543 HeNe to excite Cy3.

Quantitative analysis of white pulp area of the spleen was determined using Image J software (NIH), as previously described (**Carnevale D et al. Nat Comm 2016**).

3.9   <u>Statistical analyses:</u>

Data are presented as mean $\pm$ SD. Statistical analyses were performed accordingly to the specific experimental design. P values were considered significant from $p<0.05$. All statistical analyses were conducted with Graphpad Prism software 7.0.

4. Results.

4.1   Generation of diet-induced obesity mouse model.

The first aim of this work was to generate a mouse model of diet-induced obesity.
To achieve this goal, C57BL/6J mice starting from 6 weeks of age were fed with a high-fat diet (HFD) or a low-fat diet (LFD) as control for a time period of 8 weeks. At the beginning of the diet protocol, all mice weight ranged around 20 grams. Since the first weeks of HFD, mice became heavier and at the third week difference were already significant when compared to LFD controls mice, reaching around 30 grams at the eighth week of diet procedure. The difference between mice fed with the two diets were even more clear analyzing weekly body weight increment. The percentage increment in body weight was significant since the first week of diet (**Figure 10**).
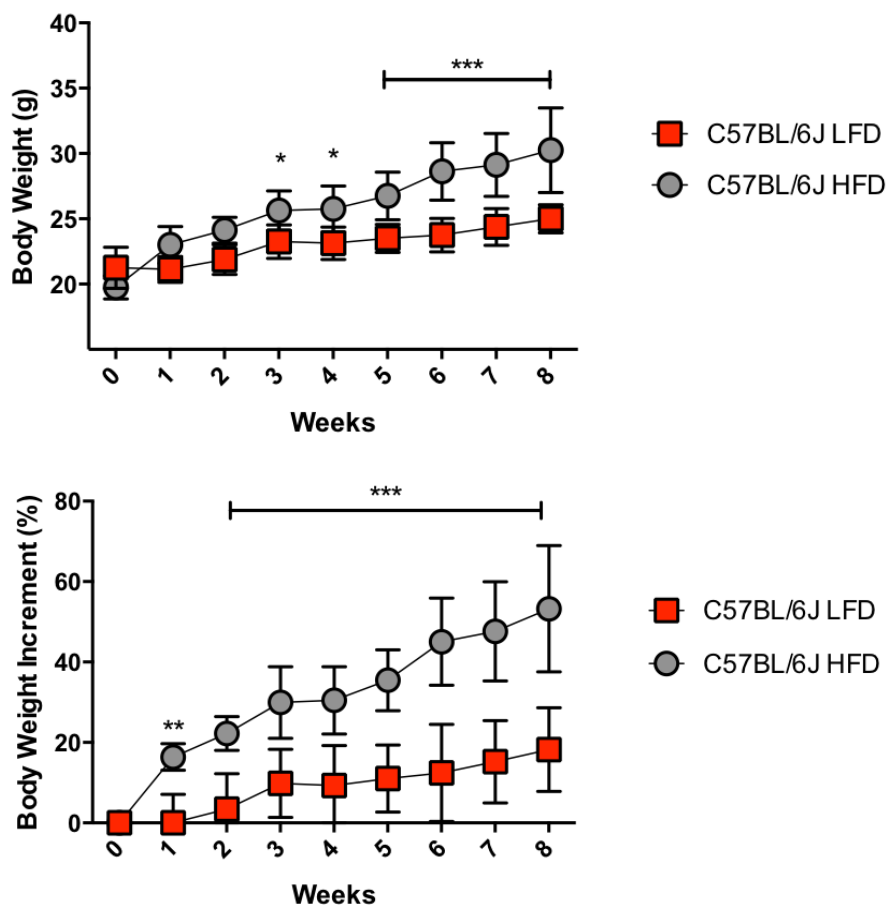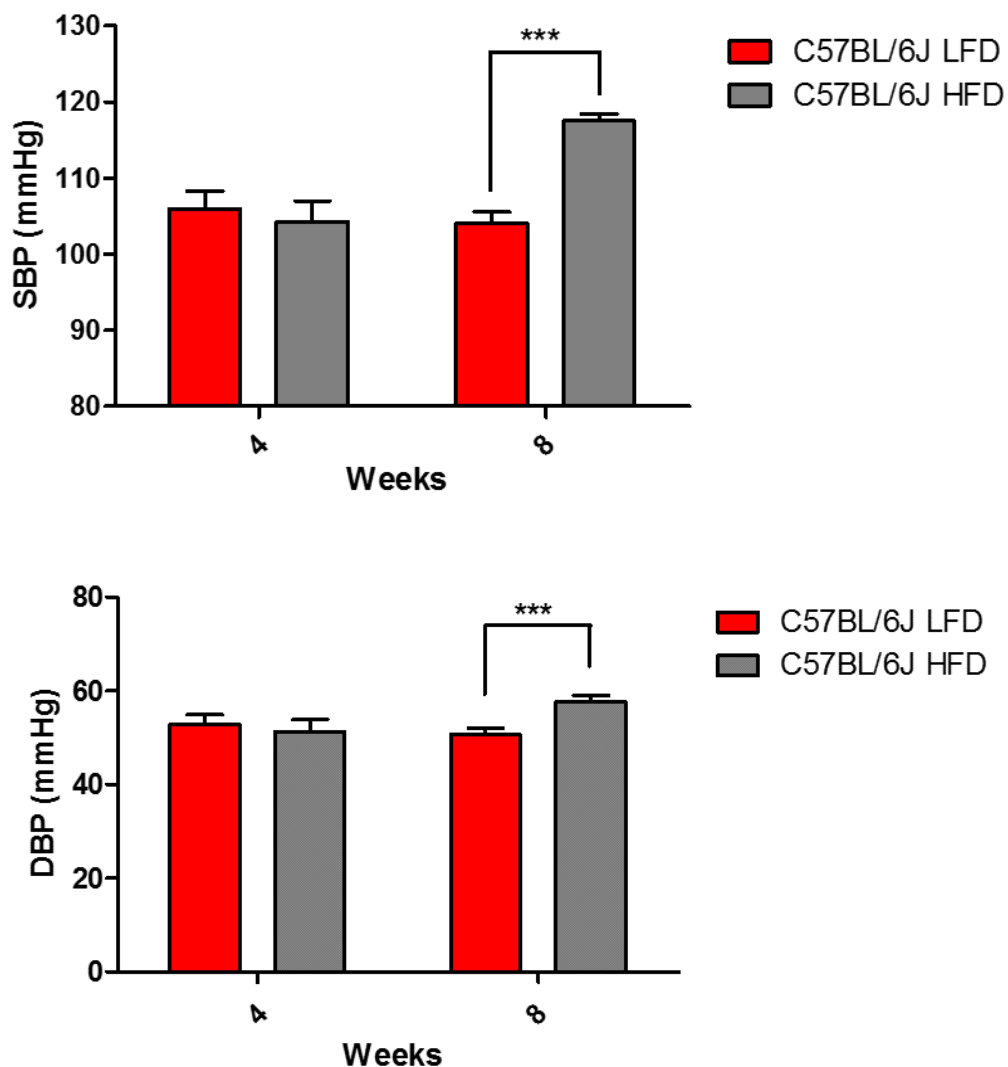

Figure 10: Body weight and Body weight increment in C57BL/6J mice fed with a HFD or a LFD for 8 weeks. Panel represent mean ± SD. (*** p<0.001 vs LFD controls), (n=8 per group).

In order to assess if HFD-induced obesity was able to promote the onset of hypertension, mice were analyzed for blood pressure increase with non-invasive tail cuff system.

Blood pressure was monitored every 4 weeks since the beginning of the diet protocol and we observed that at the eighth week C57BL/6J mice systolic blood pressure (SBP) was significantly increased compared to LFD-fed control mice. The same evidence was observed for diastolic blood pressure (DBP) (**Figure 11**).

Figure 11: Blood pressure measurements: SBP and DBP in C57BL/6J mice fed with a HFD or a LFD for 8 weeks. Panel represent mean ± SD. (*** $p<0.001$ vs LFD controls), (n=8 per group).

Overall these data demonstrate that C57BL/6J mice upon HFD become significantly obese as compared to LFD-fed mice and importantly, mice develop blood pressure rise already after 8 weeks of HFD-feeding.

4.2 Diet-induced obesity induces Splenic Sympathetic Nervous System activation.

A great amount of studies has pointed out the relevance of Sympathetic Nervous System (SNS) activation in response to obesity (**Hall JE et al. Circ Res 2015**) and other hypertensive stimuli, as Angiotensin II and Deoxycorticosterone acetate (DOCA)-Salt (**Carnevale et al. Immunity 2014, Carnevale et al. Nat Comm 2016**), promoting the increase of blood pressure level.

Once demonstrated the reliability of our mouse model of diet-induced obesity associated with hypertension development, the second aim was to explore the relevance of Sympathetic Nervous System at splenic district. To assess if HFD-feeding promotes increasing in Splenic Sympathetic Nerve Activity (SSNA), we performed nerve recording experiments directly on the splenic nerve in mice fed with HFD or LFD after 8 weeks of diet administration.

Sympathetic nerve discharges were recorded for a time period of 1 hour; meanwhile, blood pressure was monitored to ensure animal stability all over the nerve recording procedure.

In **Figure 12,** is shown a representative example of splenic nerve recording in both HFD-fed mice and LFD-fed controls.

We observed that HFD-fed mice had increased SSNA as compared to LFD-fed mice. Importantly, despite being under anesthesia, analysis of blood pressure monitoring in HFD-fed mice confirmed elevated levels as compared to LFD-fed mice.

Variation in SSNA was assessed with Firing Frequency and Amplitude Gain quantification in the same time window of 10 minutes for all mice. With the term Firing Frequency, we refer to the mean number of spikes that takes place in the

selected interval. Amplitude Gain, instead, refers to the mean amplitude increase of spikes occurring in the same time course. (**Figure 13**)
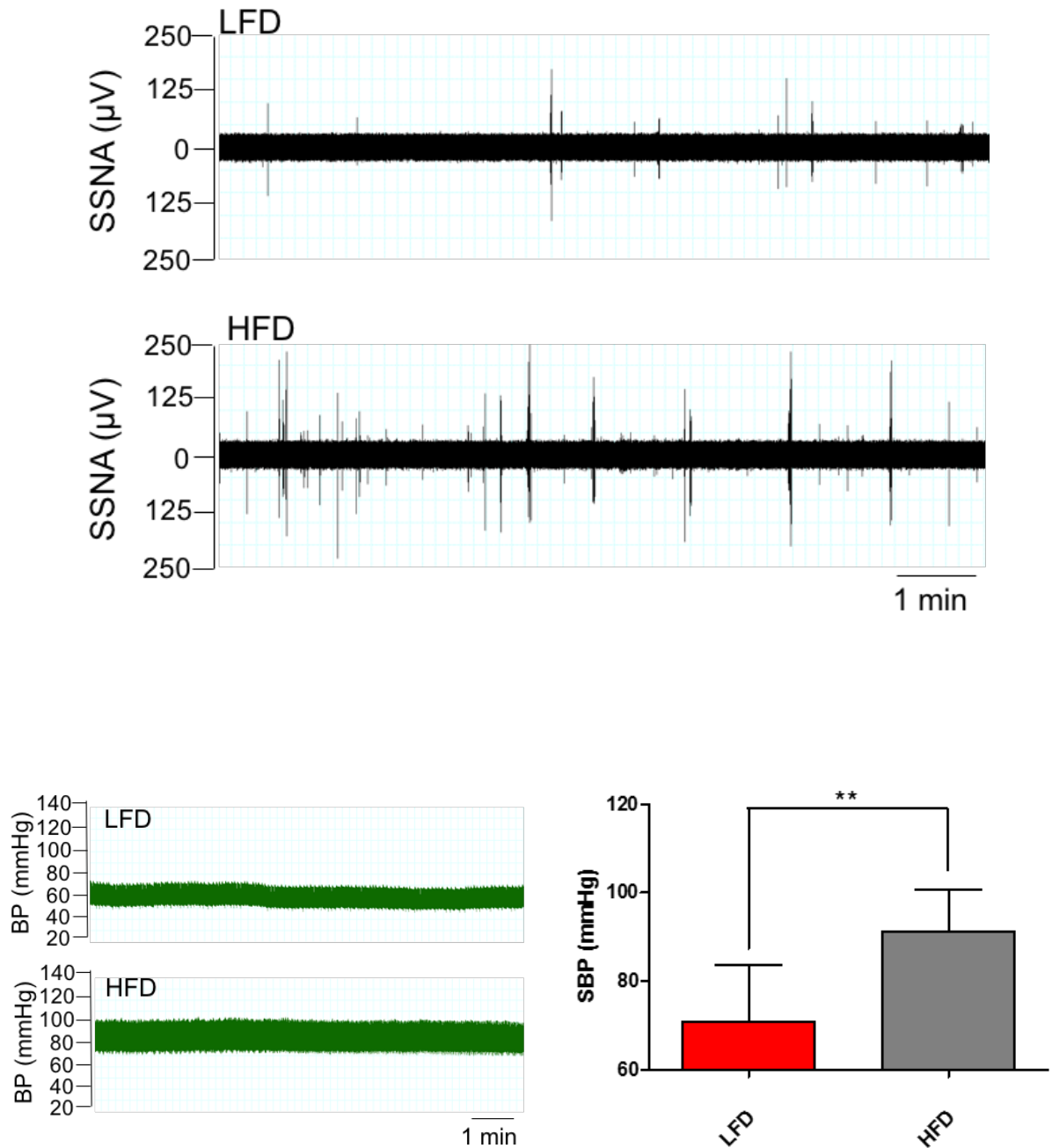

Figure 12: Representative Electrophysiological Raw signal of SSNA (upper) and simultaneous blood pressure measurements and analysis (lower) in C57BL/6J mice after 8 weeks of HFD- or LFD-feeding (LFD mice n=7 and HFD mice n=7; independent samples Student's t-test; Panels represent mean ± SD. **P<0.01, *P<0.05).
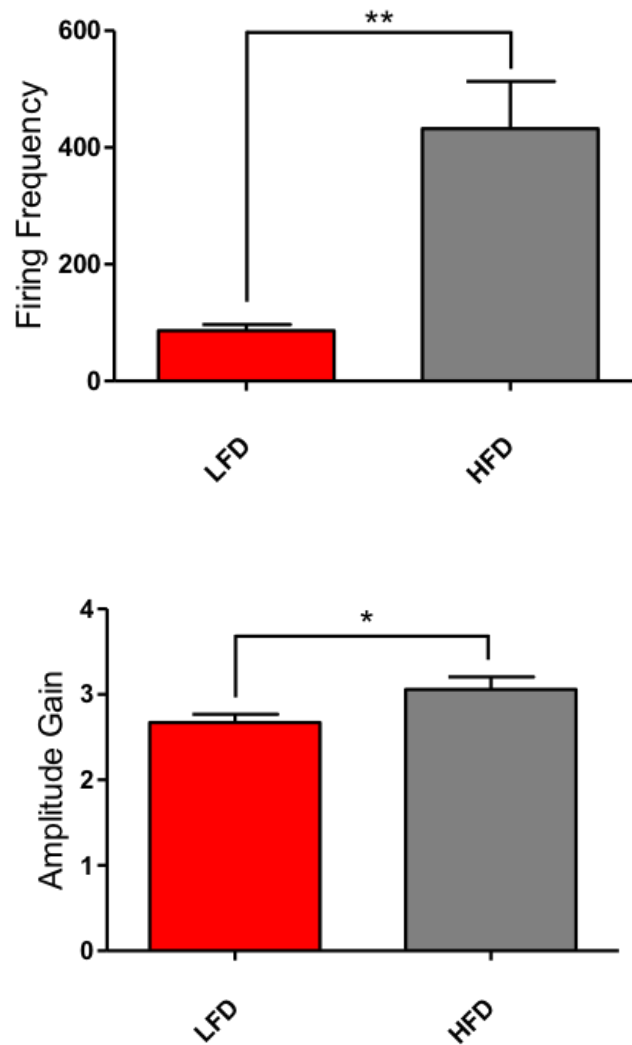
Figure 13: Electrophysiological raw analysis of Firing Frequency and Amplitude Gain in C57BL/6J mice upon LFD and HFD (LFD mice n=7 and HFD mice n=7; independent samples Student's t-test; Panels represent mean ± SD. **P<0.01, *P<0.05).

As a confirm of the increased sympathetic activity of the splenic nerve induced by HFD feeding, we performed immunostaining of tyrosine hydroxylase (TH). TH is involved in the pathway of Norepinephrine (NE) production (**Carnevale et al Immunity 2014**) that is the principal neurotransmitter released from celiac ganglion-originating nerve fibers that innervate the spleen (**Zhai et al. Crit Med Care 2017**).

We observed, indeed, that TH was highly increased in the spleen of C57BL/6J mice upon HFD. The counterstaining with CD169 expressed on macrophages surface localize TH production along the splenic Marginal Zone (MZ) (**Figure 14**).
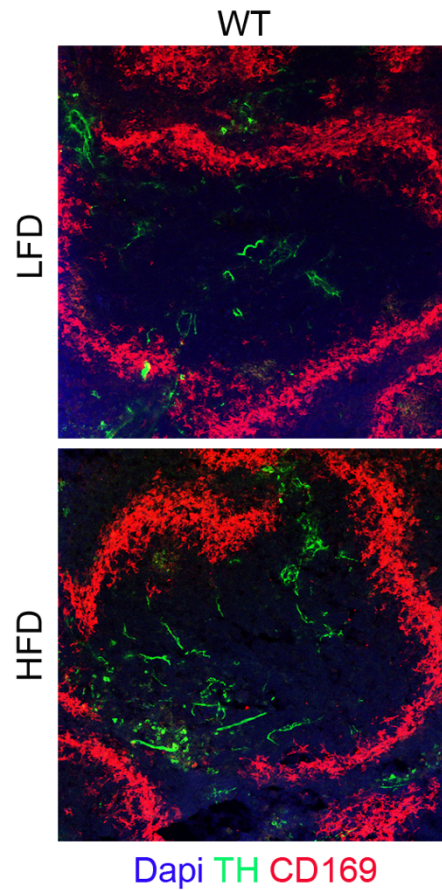
Figure 14: Immunostaining for TH in splenic MZ, showing increased expression in C57BL/6J mice upon HFD as compared to LFD-fed control mice.

Overall, these data prove that HFD feeding for 8 weeks induced significant activation of SSNA expressed as increase in both firing frequency and amplitude gain. These results support the notion that multiple metabolites have been discovered having important effects on central neural pathways modulating the activity of peripheral nervous system (**Lori et al Int J Mol Sci 2017**) eliciting hypertension.

4.3 Sympathetic nerves activity and the splenic secondary lymphoid organ do not contribute to weight gain.

In light of what we have demonstrated up to this point, we tempted to explore the causal role of the enhanced sympathetic nerve discharge toward the spleen and the relevance of the splenic organ itself in the onset of obesity and obesity induced-hypertension. On this issue, we abrogated the sympathetic innervation through specific celiac ganglionectomy (CGX) in C57BL/6J mice and subsequently mice were fed with LFD or HFD. On the other side, to investigate the relevance of the spleen, we removed the splenic organ through splenectomy.

Sham mice fed with the low-fat or the high-fat diet were used as control and the diet protocol was extended to 16 weeks to assess long-term effect of different manipulation on body weight growth pace.

Monitoring body weights weekly, we observed that both Sham, CGX and splectomized mice upon HFD became obese reaching similar body weight at the end of the 16 weeks of diet, significantly higher than control mice upon LFD (**Figure 15**).

To ensure a more accurate analysis of weight increase, data were additionally analyzed as body weight percentage of increment. Such analysis effectively demonstrated that CGX and Splenectomized mice upon HFD increased their weight significantly more as compared to Sham HFD mice (**Figure 16**).

In conclusion, these data provide important evidence that despite sympathetic activity is enhanced during the development of obesity, it does not play any role in weight accumulation processes since neither celiac ganglionectomy nor spleen surgical removal could prevent diet-induced obesity.
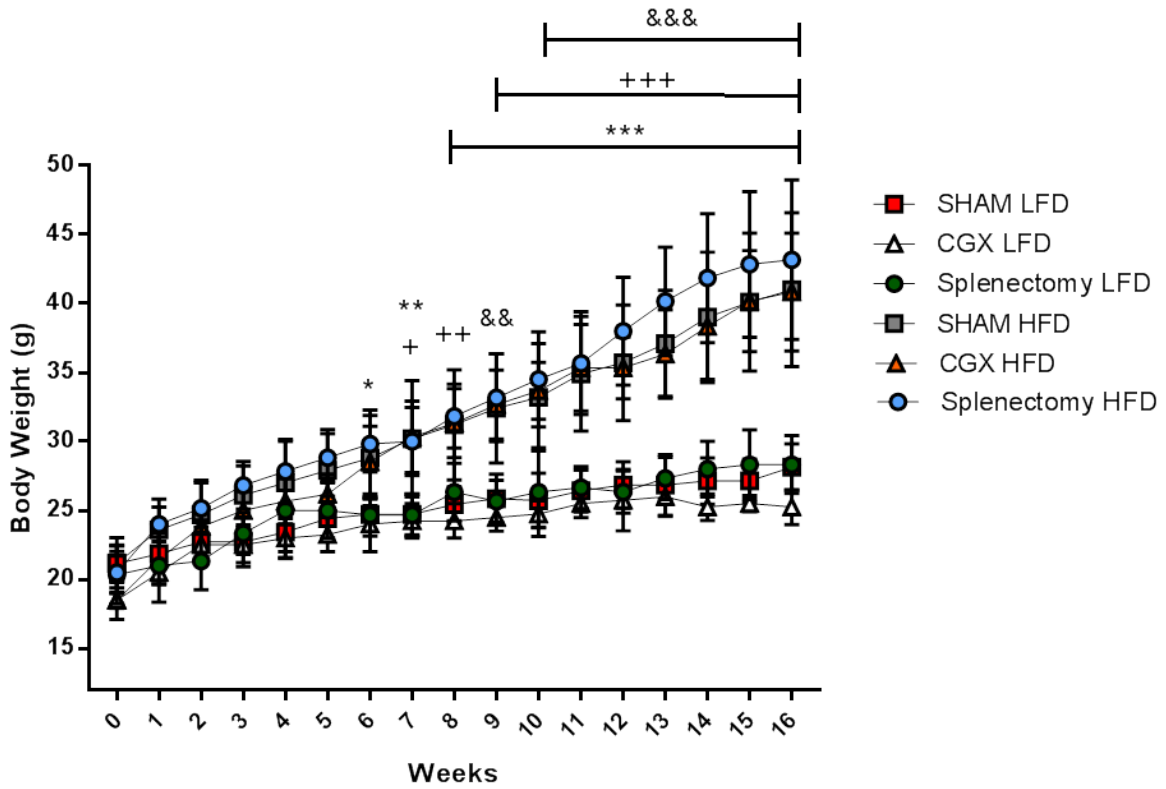
Figure 15: Body weight measurement of Sham, CGX and splenectomized mice upon HFD and LFD. (Sham LFD n=7, CGX LFD n=4, Splenectomy LFD n=3, Sham HFD n=10, CGX HFD n=6, Splenectomy HFD n=6; two-way ANOVA followed by Tukey post-hoc test; Panels represent mean ± DS. *P<0.05, **P<0.01, ***P<0.001, ++P<0.01, +++P<0.001, &&P<0.01, &&&P<0.001).
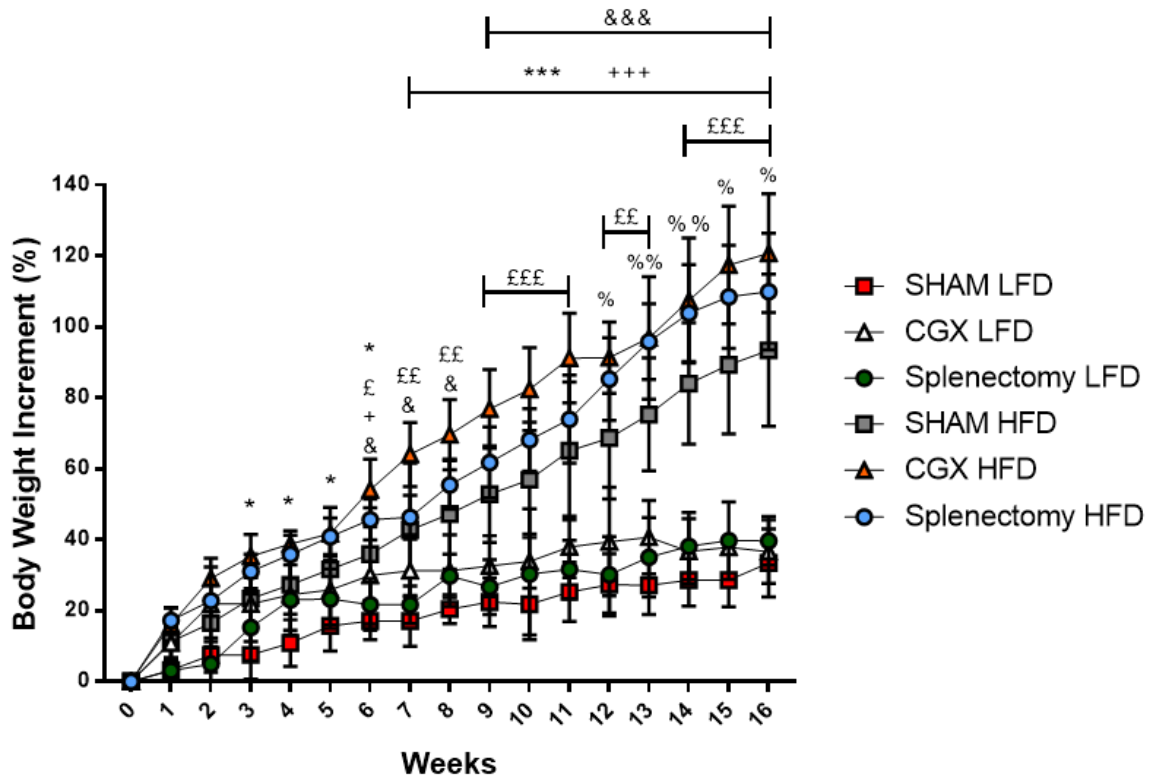
Figure 16: Body weight increase of Sham, CGX and splenectomized mice upon HFD and LFD. (Sham LFD n=7, CGX LFD n=4, Splenectomy LFD n=3, Sham HFD n=10, CGX HFD n=6, Splenectomy HFD n=6; two-way ANOVA followed by Tukey post-hoc test; Panels represent mean ± DS. *P<0.05, **P<0.01, ***P<0.001, +P<0.05, ++P<0.01, +++P<0.001, &P<0.05 &&P<0.01, &&&P<0.001; £P<0.05 ££P<0.01, £££P<0.001; %P<0.05 %%P<0.01).

Progressively weight measurement is the simplest way to determine weight increase, but it does not allow to appreciate any difference in fat deposition all over the mouse body.

Despite no difference was found in final body weight among HFD-fed mice groups, to investigate if splenic sympathetic communication blockade or splenectomy could differentially influence fat accumulation, immediately after the sixteenth week of diet protocol, we performed a non-invasive quantitation of body fat mass composition in both obese and lean Sham, CGX and Splenectomized mice (**Figure 17**).
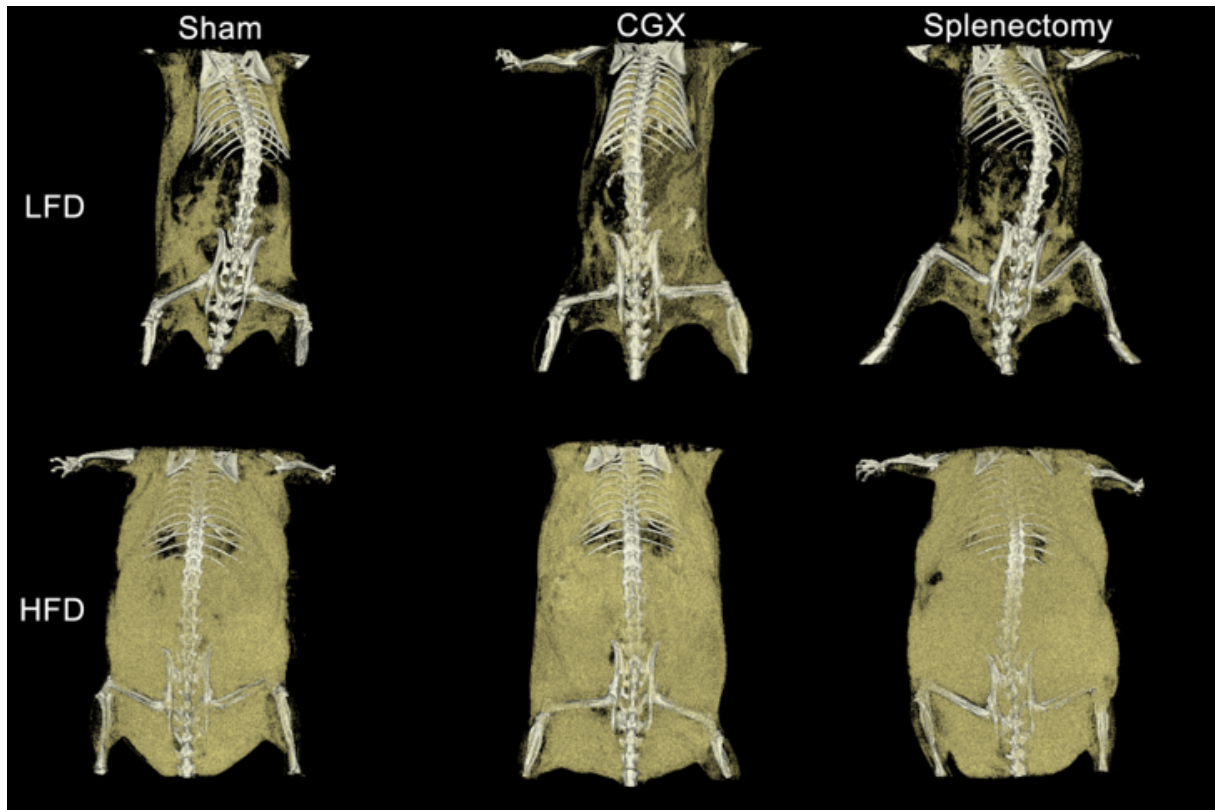
Figure 17: Representative whole-body micro-computed tomography (MicroCT) images of Sham, CGX and Splenectomized mice upon LFD or HFD after 16 weeks of diet administration.

Whole-body micro-computed tomography (MicroCT) images do not revealed any differential accumulation of fat depots in lean or obese mice in response to surgical procedure as compared to sham controls.

Quantitative analysis of body fat increase, was based on total fat volume on total body volume ratio. Results clearly confirmed the similarity between obese Sham, CGX and Splenectomized mice and the significant difference when compared to lean control mice (**Figure 18**).
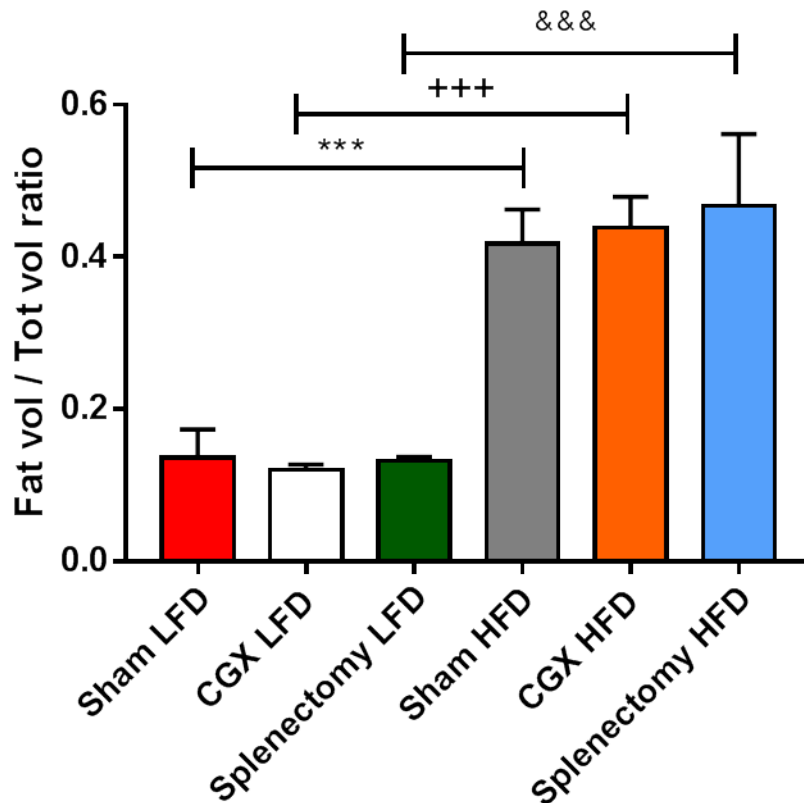
Figure 18: Quantitative analysis of fat pat increase in LFD- and HFD-fed Sham, CGX and Splenectomized mice (Sham LFD n=7, CGX LFD n=4, Splenectomy LFD n=3, Sham HFD n=10, CGX HFD n=6, Splenectomy HFD n=6) (Panel represent mean ± SD) (***, +++, &&& P<0.001).

4.4 Despite developing obesity, CGX and Splenectomized mice are protected against hypertension.

In order to evaluate if sympathetic signal transmission pathway to the spleen or the spleen itself, could contribute to the onset of hypertension upon HFD, in parallel to weekly body weight assessment, non-invasive tail-cuff blood pressure measurements were performed every 4 weeks from the start of the diet protocol.

As expected from weight gain, Sham mice blood pressure increased significantly starting at the second month of high-fat feeding. Surprisingly, both CGX and splenectomized mice, despite becoming obese were protected against hypertension. Systolic blood pressure of CGX and splenectomized mice indeed, completely

overlapped that of all LFD-fed mice groups being significantly lower than that of Sham HFD mice (**Figure 19**).
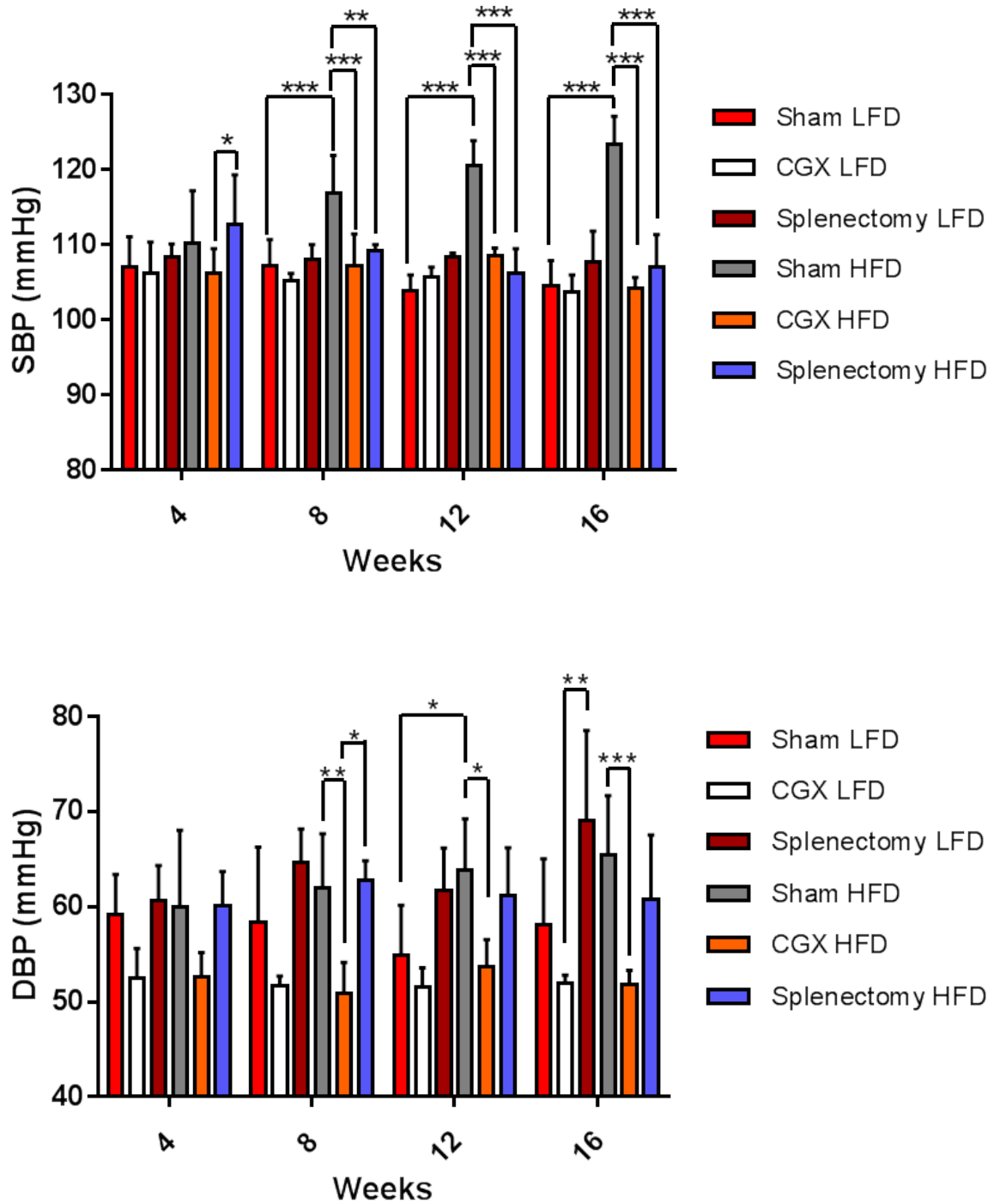
Figure 19: Non-invasive tail cuff blood pressure measurements assessed every 4 weeks from the beginning of the diet procedure. (Sham LFD n=7, CGX LFD n=4, Splenectomy LFD n=3,

Sham HFD n=10, CGX HFD n=6, Splenectomy HFD n=6) (two-way ANOVA followed by Tukey post-hoc test; Panels represent mean ± SD.) (*<0.05, **P<0.01, ***P<0.001)

Collectively, these data effectively demonstrate that surgical procedure to interrupt neural sympathetic pathway toward the spleen or to remove the splenic organ do not affect obesity development. On the contrary, the hypertensive phenotype evoked by a challenge as diet-induced obesity, was completely blunted in celiac ganglionectomized and splenectomized mice.

4.5 PlGF expression in the spleen is enhanced in response to SSNA

Recent finding revealed that the spleen is the site that hosts a neuro-immune interaction in response to hypertensive stimuli as Angiotensin II or DOCA-salt that is critically supervised by PlGF (**Carnevale et al., Immunity 2014, Carnevale et al. Nat com 2016**). In particular, splenic PlGF was found overexpressed when SSNA is enhanced. The evidence highlighted by our mouse model in which diet-induced obesity increased SSNA, and the fact that inflammation is a central feature not only in hypertension but also in metabolic diseases despite its origin is not fully understand, prompted us to investigate if PlGF expression could be modulated in diet-induced obesity.

For that reason, we firstly explored the differential expression of the PlGF in response to the different diets administration after the second month of diet protocol.
Immunostaining of PlGF WT mice demonstrated that the expression of the growth factor was increased in the spleen of obese HFD-fed mice as compared to lean LFD-fed control mice. Moreover, PlGF was especially expressed along the splenic marginal zone (MZ) since it co-localized with ER-TR7 antigen present on the stromal cell surface (**Figure 20**). As expected, spleen of PlGF KO mice was completely devoid of

PlGF staining. Quantitative analysis revealed that in HFD-fed mice, PlGF was produced more than twice as compared to LFD-fed mice (**Figure 20**).

These evidences suggested that PlGF production along the marginal zone is affected by diet-induced obesity, thus, we aimed to explore the possible contribution of PlGF to diet-induced obesity and related hypertension development.
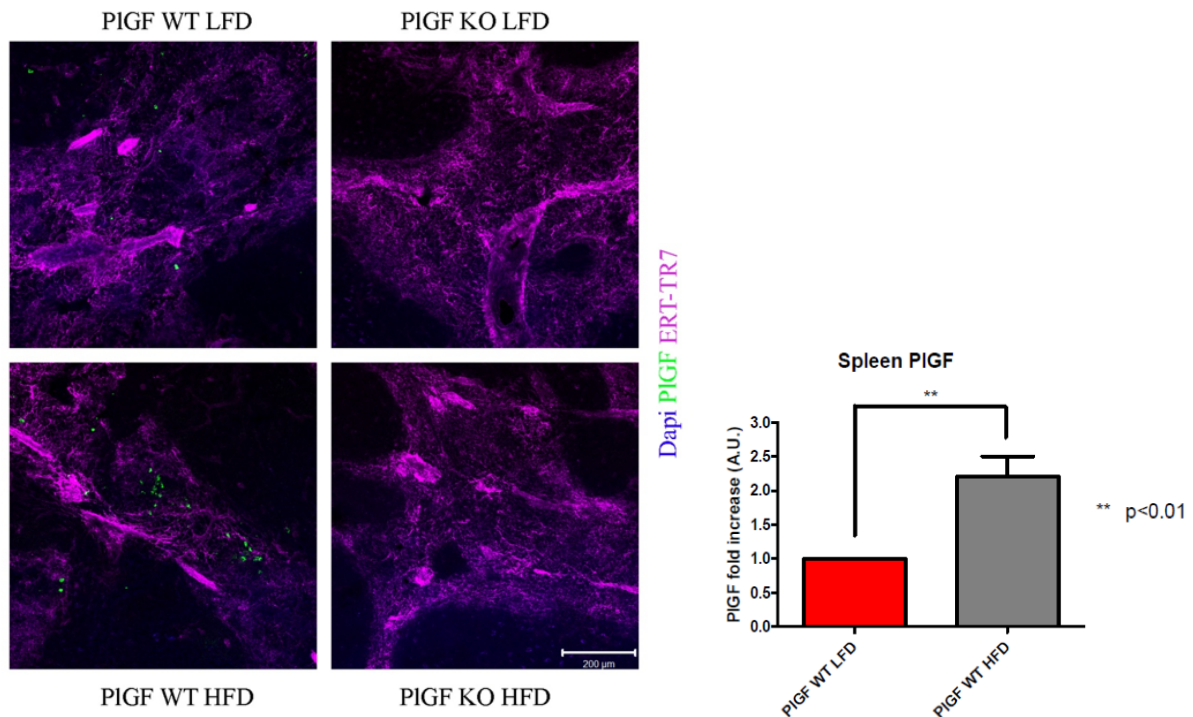
Figure 20: Representative immunostaining and relative quantification, expressed as fold of increase, of PlGF (green) expression in the spleen counterstained with ER-TR7 (red) identifying marginal zone stromal cells, in PlGF WT and KO mice upon LFD and HFD. (PlGF WT LFD n=8, PlGF KO LFD n=4, PlGF WT HFD n=5, PlGF KO HFD n=3) (T-test, Panel represent mean ± SD; ** P<0.01)

4.6 PlGF do not modulate weight gain upon HFD but plays a pivotal role in blood pressure increase.

In order to investigate if PlGF plays a role in diet-induced obesity development we administered LFD and HFD to 6 weeks old PlGF WT and PlGF KO mice for 16 weeks

following the same procedure as previously described for Sham, CGX and splenectomized mice.

Body weight data showed that at the end of the sixteenth week, both PlGF WT and PlGF KO mice become obese, increasing significantly their body mass as compared to respective LFD-fed control mice. In particular, no statistical difference was detected among the two genotypes upon both diets at the end of the sixteen weeks and the growth rate appeared to be similar (**Figure 21**).

*	PlGF WT LFD vs PlGF WT HFD
#	PlGF KO LFD vs PlGF KO HFD
§	PlGF WT HFD vs PlGF KO HFD
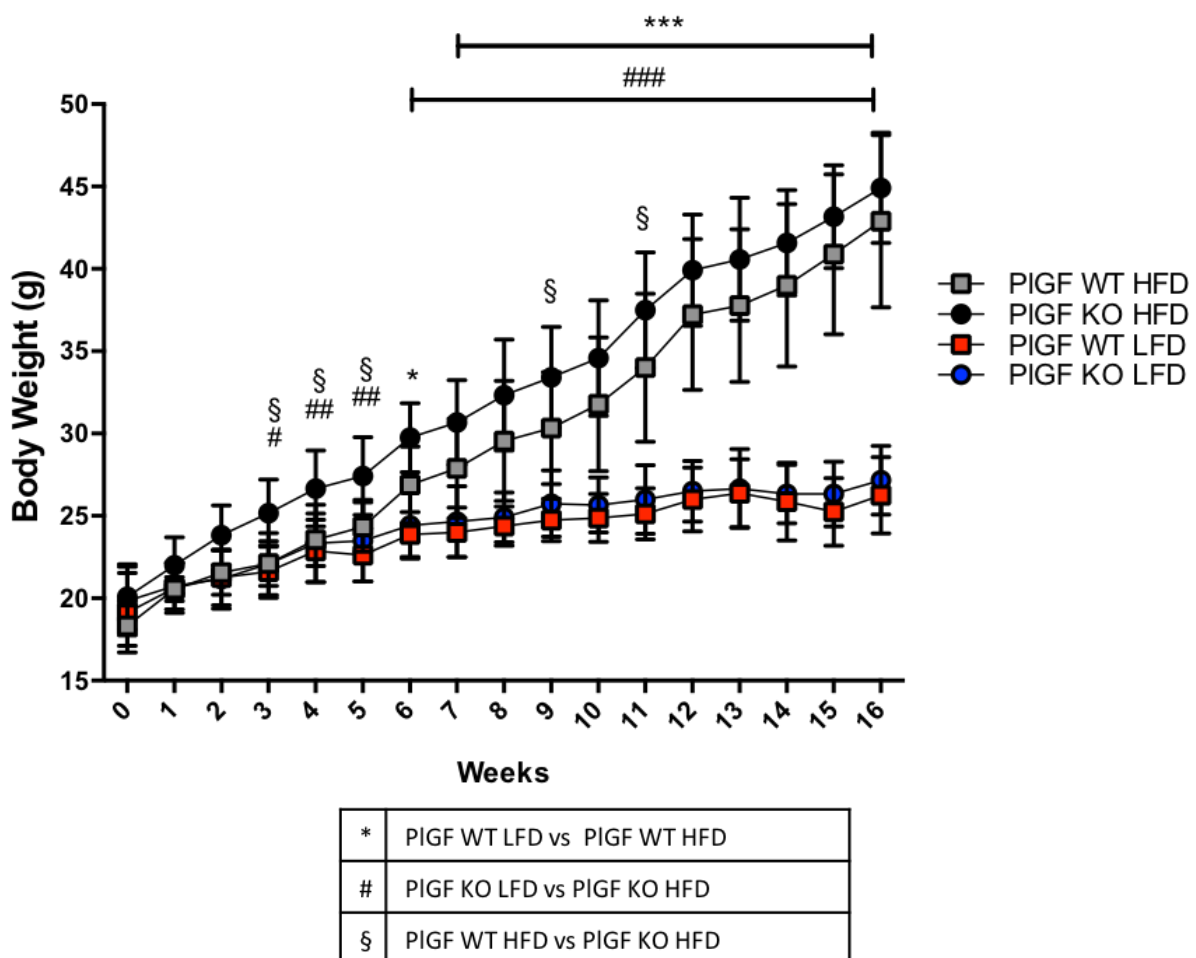
Figure 21: Body weight measurement during the entire diet procedure for PlGF WT and PlGF KO mice upon LFD and HFD. (PlGF WT LFD n=8, PlGF KO LFD n=12, PlGF WT HFD n=9, PlGF KO HFD n=12) (two-way ANOVA followed by Tukey post-hoc test; Panels represent mean ± SD; *, #,§ $P<0.05$, **, §§$P<0.01$, ***$P<0.001$)

Analyses of body weight percentage increment, clearly showed that the growth rate of PlGF WT and PlGF KO mice upon HFD was completely overlapping for each week during the entire diet protocols and the same trend affected control mice upon LFD (**Figure 22**). At the end of the diet procedure all PlGF WT and PlGF KO mice were assessed for fat pad accumulation with non-invasive microCT (**Figure 23**).
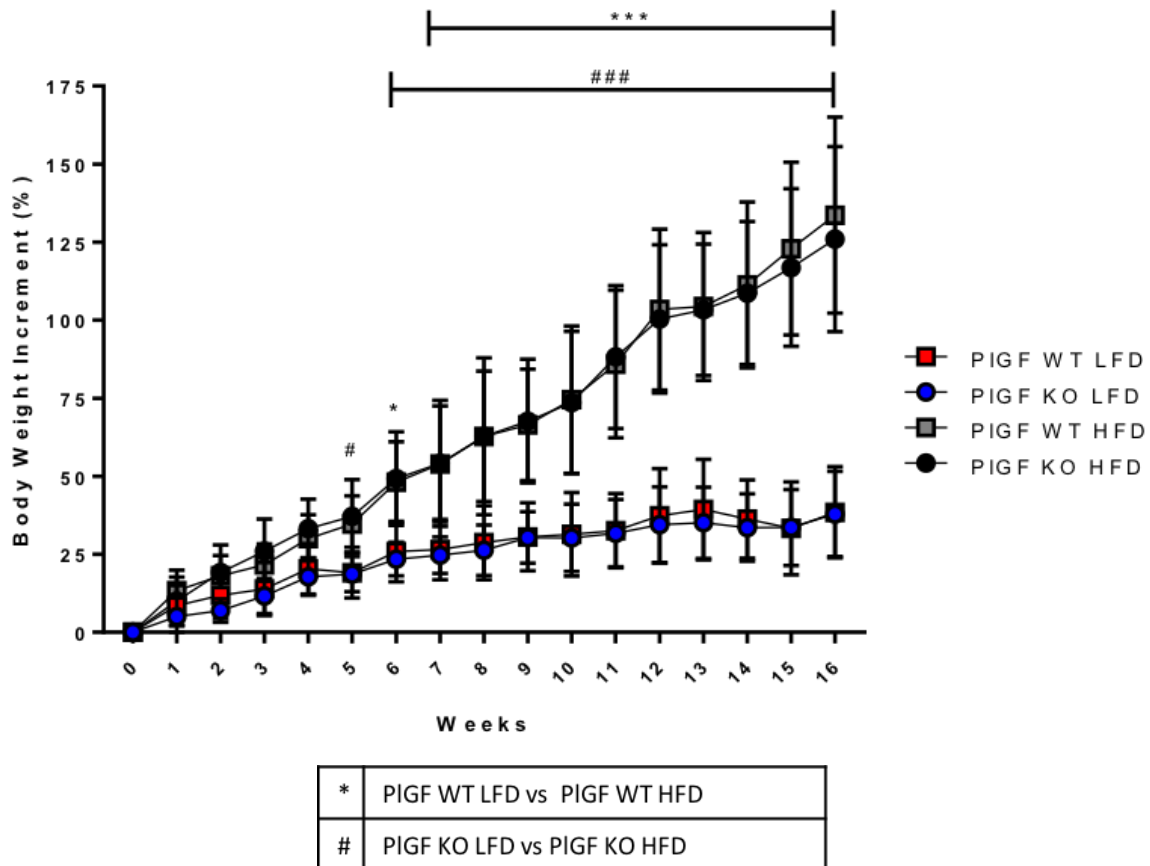
Figure 22: PlGF WT and PlGF KO mice body weight percentage increase upon LFD or HFD during the diet protocol. (PlGF WT LFD n=8, PlGF KO LFD n=12, PlGF WT HFD n=9, PlGF KO HFD n=12) (Two-way ANOVA followed by Tukey post-hoc test; Panels represent mean ± SD; *,#P<0.05, **P<0.01, ***P<0.001).
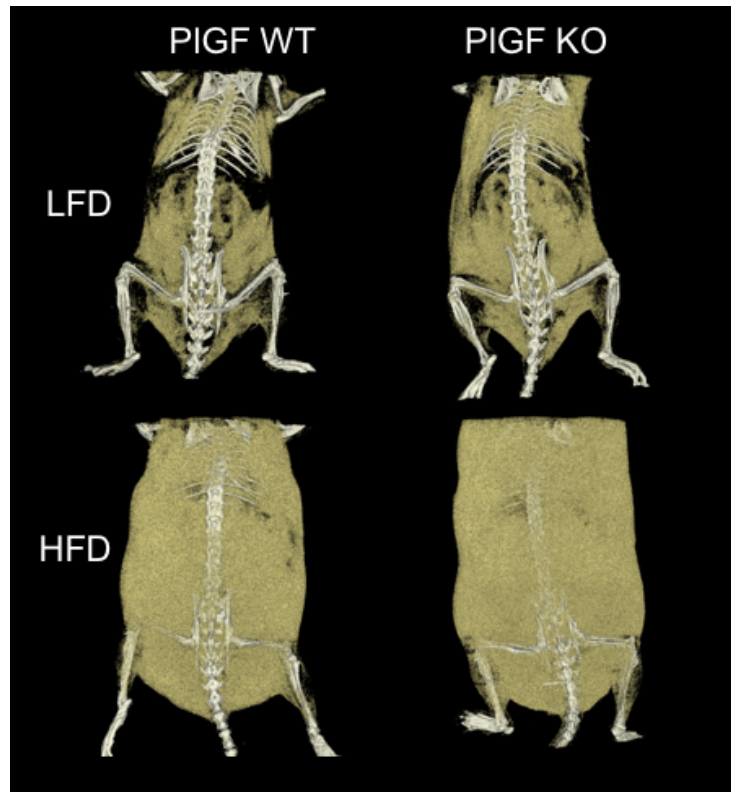
Figure 23: Represesentative microCT comparison of fat pad volume in PlGF WT and PlGF KO mice fed with LFD and HFD.

Quantitative analysis of fat pad volume confirmed the presence of an equal amount of adipose tissue volume between the two genotypes when fed HFD or LFD (**Figure 24**).
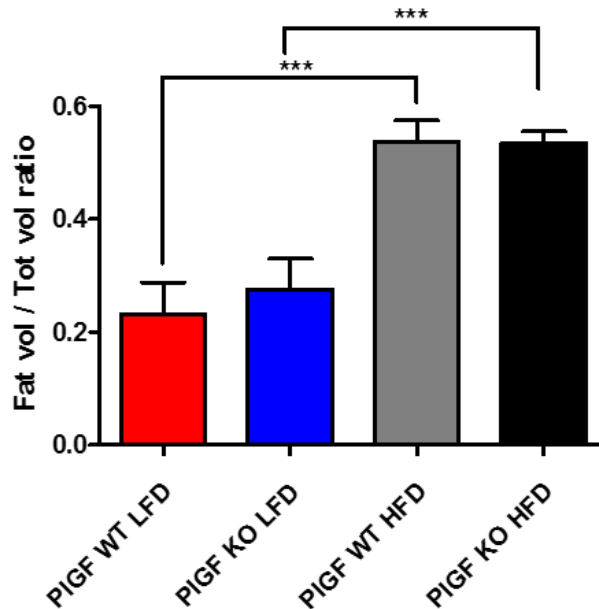
Figure 24: MicroCT analysis of fat pad volume on total body volume ratio of PlGF WT and PlGF KO mice fed with LFD and HFD. (PlGF WT LFD n=8, PlGF KO LFD n=12, PlGF WT HFD n=9, PlGF KO HFD n=12) (two-way ANOVA followed by Tukey post-hoc test; Panels represent mean ± SD; ***P<0.001).

PlGF seems not to be a key regulator of accumulation in fat pad during obesity resembling data observed for CGX and splenectomized mice.

Conversely, blood pressure analysis provided important evidence. Following the same procedure, blood pressure was monitored every 4 weeks since the start of the diet protocol. Despite PlGF WT and PlGF KO became obese on HFD, reaching similar body weight, PlGF deficiency conferred protection from obesity-induced hypertension. Both systolic- and diastolic blood pressure of HFD-fed PlGF KO mice was comparable to LFD controls (**Figure 25**). HFD-fed PlGF WT mice, instead, showed a really slight but significant increase in SBP already at fourth week of diet. A more significative increase in blood pressure is evident from the twelfth week of diet as observed for DBP.
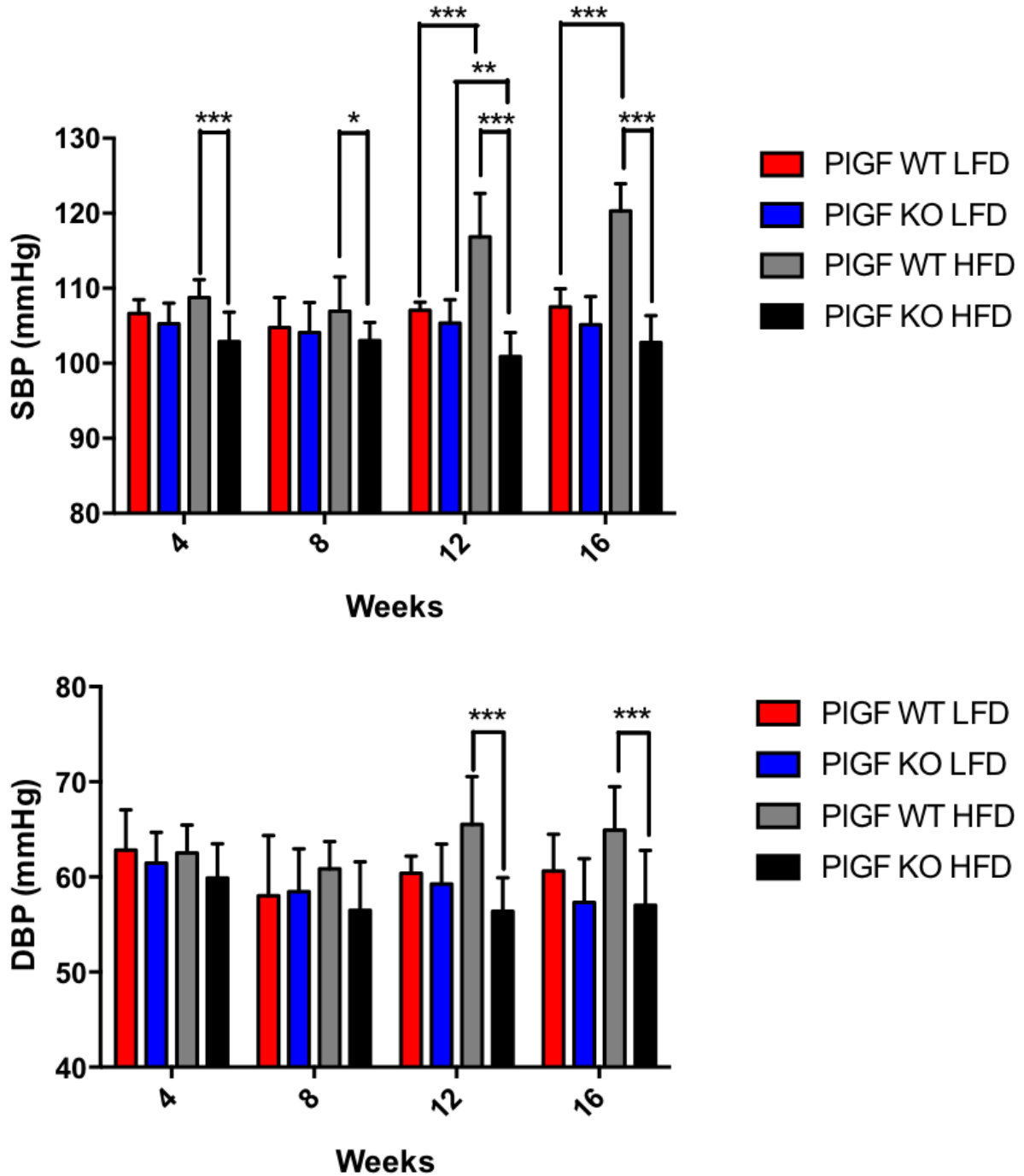
Figure 25: Non-invasive tail-cuff systolic- (upper) and diastolic (lower) blood pressure analysis of PlGF WT and PlGF KO mice upon LFD and HFD. (PlGF WT LFD n=8, PlGF KO LFD n=12, PlGF WT HFD n=9, PlGF KO HFD n=12) (two-way ANOVA followed by Tukey post-hoc test; Panels represent mean ± SD; *P<0.05, **p<0.001, ***P<0.001).

These data demonstrate that PlGF-deficiency do not influence body fat mass accumulation. The most impressive result is that, despite obesity, PlGF-deficiency confers protection against hypertension.

4.7 PlGF modulates lymphocytes egression from the spleen of mice upon HFD.

Despite the involvement of immune cells in obesity is widely described, to date the origin of inflammation has not been fully understood. Starting from the recent discovery that PlGF is not only an angiogenic factor but plays an indispensable function activating immune cells from the splenic reservoir promoting their migration toward target organs of hypertension (**Carnevale D et al. Immunity 2014**), we hypothesized that the splenic PlGF could affect such phenotype in obesity. To confirm our hypothesis, we performed immunostaining of splenic CD3 positive cells, residing in the splenic white pulp, counterstained with B220 that delineate the splenic red pulp.

Quantitative Image J analysis demonstrated that HFD-fed PlGF WT CD3 positive area was significantly smaller than that of PlGF KO mice upon HFD as well as when compared to LFD control mice (**Figure 26).**
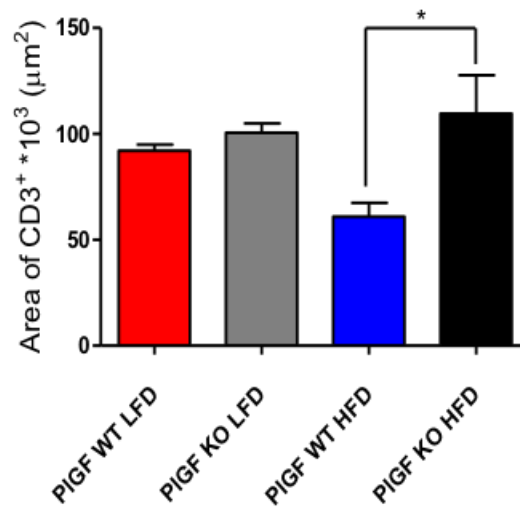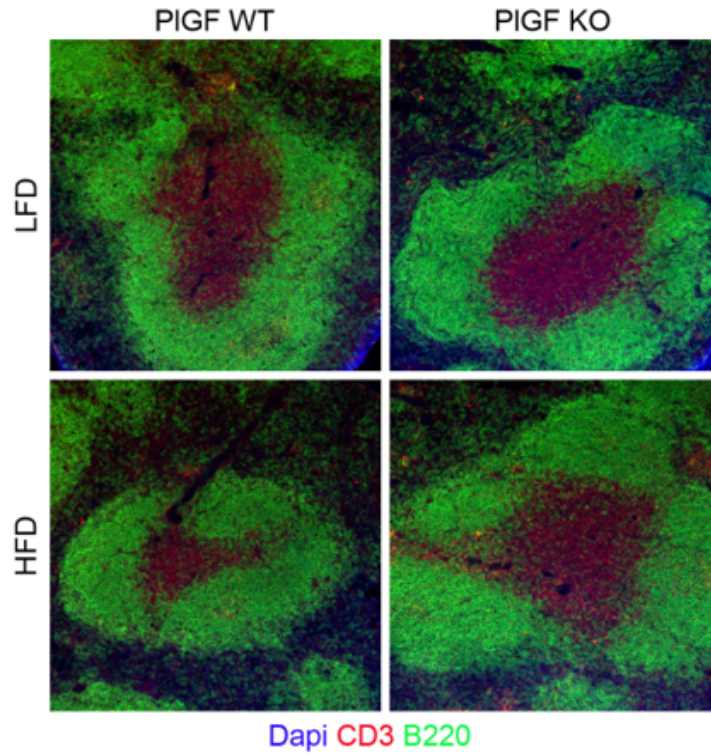
Figure 26: Representative images of splenic immunostaining, showing CD3 positive white pulp counterstained with red pulp B220 antigen and quantitative analysis of CD3 positive white pulp area. (two-way ANOVA followed by Tukey post-hoc test; Panels represent mean ± DS; *$P<0.05$)

5. Discussion.

A great amount of evidence supports the notion that an uncontrolled increase in body weight results in higher risk to develop obesity with the outbreak of extremely detrimental comorbidities, among which cardiovascular diseases. Nevertheless, the relevance of many pathophysiological contribution remains, to date, not completely understood.

Results obtained in this work provide significant insights in the development of hypertension induced by obesity. In particular we demonstrate a pivotal role, previously unknown, played by the SNS at the splenic district, the spleen and the PlGF contributing to blood pressure rise during the onset of obesity.

The first goal of this work was to develop an effective mouse model of diet induced obesity through the administration of HFD. Effectively it has been observed that HFD fed mice became significantly heavier than control mice upon LFD. Together, blood pressure analysis showed that HFD-feeding enhanced significantly blood pressure since the eighth week from the beginning of the diet procedure confirming that HFD is an important hypertensive challenge promoting the onset of cardiovascular disease (**Simonds SE et al. Cell 2014**).

Once the murine model of diet-induced obesity and related hypertension was generated, the second aim of this study was to explore if sympathetic nerve drive was modulated especially in the splenic district in response to diet-induced obesity.

It is widely recognized that the sympathetic activation induces and maintain blood pressure increase (**Hall JE Hypertension 2003; Hall JE et al. Am J Hypertens 2001; Esler M Am J Hypertension 2001; Malpas SC Physiol rev 2010**) in response to different hypertensive challenges as Angiotensin II or DOCA-salt determining end-organ damage (**Carnevale D et al. Immunity 2014; Carnevale D et al. Nat comm 2016**). Likewise, it has been characterized the relationship between obesity-induced hypertension and renal sympathetic nerve activation suggesting that renal nerve intervention could be a promising strategy to counteract hypertension (**Rahmouni K et al. Hypertension 2005; Asirvatham-jeyaraj N et al. Hypertension 2016**). Clinical

trials aiming at assessing the results of renal artery denervation treatment in hypertensive subject provided conflicting evidences (**Esler MD et al. Eur Heart J 2014; Bhatt DL N Engl J Med 2014**). Such ineffective results open up the possibility that different sympathetic mechanism, other than the renal one, could be involved in the onset of obesity-induced hypertension.

The activation of an inflammatory response is a common feature that is shared between obesity and hypertension (**McLaughlin T J Clin Invest 2017; Madhur MS and Harrison DG Circ Res 2012**). The recent discovery that the spleen is a central player in the onset of hypertension where afferent sympathetic neural signals are sensed and immune cells are activated through PlGF modulation guided us in focusing our attention on splenic sympathetic nerve (**Carnevale D et al Hypertension 2014**).

For the first time ever, we performed splenic sympathetic nerve recording and revealed that diet-induced obesity sustains an increased SSNA.

This result describes a previously unknown evidence that diet-induced obesity promotes sympathetic activity through splenic innervation; opening the possibility that other sympathetic mechanism, rather than the renal one, could be involved in the onset of obesity-induced hypertension.

To assess whether the SSNA is involved in the development of diet-induced obesity or has an impact on obesity-induced hypertension or both, we moved forward exploring the effect of SSNA blockade in mice fed HFD. Celiac ganglionectomy and splenectomy did not rescue the obesity phenotype upon HFD, however, we observed that both treatments were efficient in blunting blood pressure increase despite obesity. Such result has important clinical implication for the treatment of obesity related hypertension. It could represent a new therapeutic target especially for patients to whom other surgical interventions, as renal denervation, had failed.

Recent advances in research emphasized the role of immune system in blood pressure rise in different mouse model in which hypertension was induced by Angiotensin II or DOCA-salt and the relevance of the spleen as a hub that connects

nervous and immune system through PlGF. (**Guzik TJ et al. J Exp Med 2007; Carnevale D et al. Hypertension 2014; Zhang J et al. Curr Opin Pharmacol 2015**). In this view, we found that in response to enhanced splenic sympathetic nerve discharge upon HFD, PlGF in the spleen was significantly overexpressed. That evidence prompted us to hypothesize that PlGF could be involved in the pathogenesis of diet induced obesity and related hypertension.

Our results indicate that PlGF WT and PlGF KO mice upon HFD did not differ in body weight but PlGF-deficiency protected obese mice from hypertension development.

These results are in agreement with our previous evidences observed in celiac ganglionectomized and splenectomized mice. Abrogating splenic sympathetic nerve activity pathway or the downstream PlGF expression do not impact at all on weight gain during obesity development and accordingly accumulation of fat pad is indistinguishable among HFD-fed mice. On the other side, the surgery removal of the celiac ganglion and the spleen, or the inhibition of PlGF completely protect against obesity-induced hypertension.

The lack of abundant literature concerning murine models of diet-induced obesity in PlGF mice does not allow numerous comparison. However, Lijnen and colleagues showed different results: PlGF KO mice gained significantly less body weight after 15 weeks of HFD feeding when compared to WT mice controls upon the same diet (**Lijnen HR et al. Diabetes 2006**). The discrepancies with our results probably could reside in the different mice strain adopted; in fact, they used both PlGF WT and PlGF KO mice with 50% Swiss and 50% 129SV genetic background. Differently, our PlGF mouse model background is 100% C57BL/6J. As previously described, different strains could manifest different response to HFD-induced weight gain (**Montgomery MK et al. Diabetologia 2013**).

Finally, the involvement of immunity in obesity and related disease has been widely confirmed but, exactly, the mechanism that underlies its activation and, above all, its origin has not yet been fully clarified. Preliminary results on splenic immunostaining

suggested that consequentially to its overexpression upon HFD, PlGF promotes the egression of T cells from the splenic organ, suggesting that PlGF could play a role in the egression of immune cells from the splenic reservoir contributing to the onset of metainflammation. In the same way obesity-induced PlGF expression could play a pivotal role in blood pressure increase inducing immune cells infiltration in target organs of hypertension as observed in response to other hypertensive stimuli (**Carnevale D et al. Immunity 2014**).

Future efforts are needed to confirm this evidence, exploring the immune scenario in kidneys and aorta but also in other district as adipose tissue to discriminate a role of PlGF also in metabolic triggered inflammation.

Overall, the most important evidence that emerge from our results is the existence of a splenic sympathetic nervous system / PlGF axis that is indispensable in mediating obesity induced hypertension. Splenic sympathetic nerve and PlGF appear as critical targets for future therapeutic approaches to counteract hypertension in obese subjects.

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7. Annex.

The Spleen: A Hub Connecting Nervous and Immune Systems in Cardiovascular and Metabolic Diseases

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Abstract: Metabolic disorders have been identified as major health problems affecting a large portion of the world population. In addition, obesity and insulin resistance are principal risk factors for the development of cardiovascular diseases. Altered immune responses are common features of both hypertension and obesity and, moreover, the involvement of the nervous system in the modulation of immune system is gaining even more attention in both pathophysiological contexts. For these reasons, during the last decades, researches focused their efforts on the comprehension of the molecular mechanisms connecting immune system to cardiovascular and metabolic diseases. On the other hand, it has been reported that in these pathological conditions, central neural pathways modulate the activity of the peripheral nervous system, which is strongly involved in onset and progression of the disease. It is interesting to notice that neural reflex can also participate in the modulation of immune functions. In this scenario, the spleen becomes the crucial hub allowing the interaction of different systems differently involved in metabolic and cardiovascular diseases. Here, we summarize the major findings that dissect the role of the immune system in disorders related to metabolic and cardiovascular dysfunctions, and how this could also be influenced by neural reflexes.

**Keywords:** obesity; hypertension; splenic immune system; neuroimmune interactions; autonomic nervous system

1. Introduction

Despite initially having been depicted simply as a blood filter, the spleen is gaining more attention for the pivotal role played in modulating immune responses [1]. The spleen is enclosed by an elastic and fibrous capsule with emerging trabeculae directed toward the internal parenchyma, ensuring the stability and maintenance of a well-organized structure [2,3]. The central arterioles that depart from the splenic artery branch in the trabeculae to subsequently dive in the White Pulp (WP) area, which contains B cells in the follicles and T cells. At this point, blood flows through the Marginal Zone (MZ) that surrounds the WP, toward the Red Pulp (RP) area, where blood is directed to the venous sinuses (Figure 1). Then, blood flows again through the trabecular area and is conveyed to the splenic vein. The splenic RP oversees the blood filtration process, in which resident macrophages phagocyte old erythrocytes, activate response to bacterial infiltration and allow iron recycling [1].

Another important activity that takes place in the RP is the production of antibodies. Indeed, plasmablasts differentiate in response to a specific antigen within the WP. Plasmablasts show enhanced production of CXC-chemokine receptor 4 (CXCR4), which specifically binds CXC-chemokine ligand 12 (CXCL12), which is produced in large quantities in the RP and is responsible for the migration and maintenance of cells in that splenic zone. This migration is also permitted due to the

simultaneous repression of CXCR5 and CCR7 receptors on plasmablasts and lower response to the chemokines responsible for the homing in the WP follicles and T cell zone [3,4].
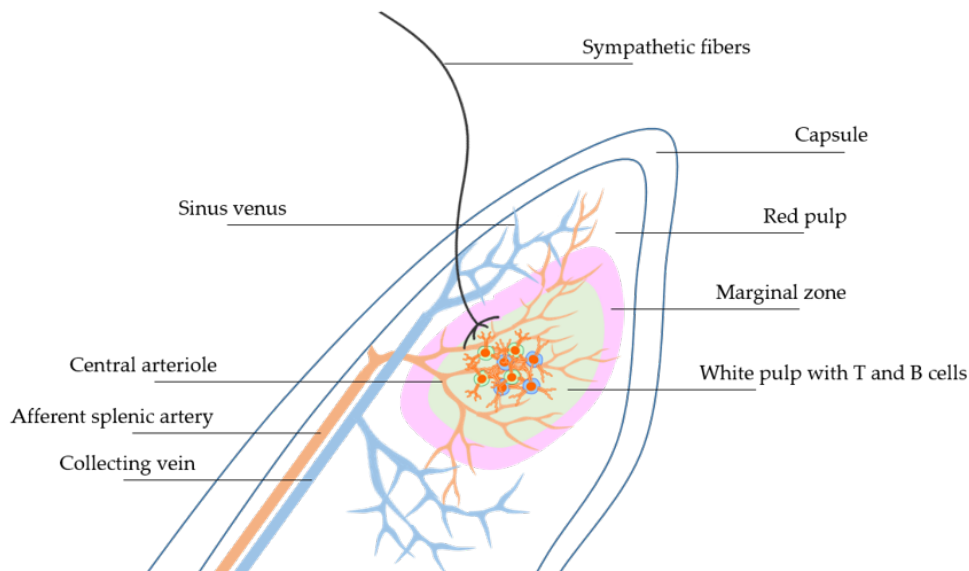
Figure 1. Schematic description of the architectural organization of splenic areas containing various immune cells: White Pulp (WP) and Red Pulp (RP) with Sympathetic nerve fibers innervating Marginal Zones (MZ).

Splenic WP is a lymphoid area in which the specific B cell receptor CXCR5 is responsible for the organization of B cell follicles, where B cells are differentiated. T cells and Dendritic Cells (DCs) expressing the CCR7 receptor are attracted and confined in the T cell zone by the CC-chemokine ligands CCL19 and CCL21 [3,5]. Mutations that abolish the expression of these chemokines and receptors determine a completely aberrant distribution of the immune cells in the correct splenic districts [5].

Finally, the MZ, separating White from RPs, represents a connection between circulating blood and immune cells. This architecture allows B cells to defend the organism from blood-borne pathogens, mainly generating different lines of immunoglobulins (IgA, IgM and IgG) [6]. Alternatively, MZ B cells are able to activate CD4+ T cells to become antigen presenting cells (APCs) [7]. Populations of immune cells present in the MZ are not limited to B cells. Indeed, subsets of Neutrophils [6], Dendritic Cells [8] and Macrophages [9] can be found in the same area of the spleen. In order to maintain the correct separation between the MZ populations and the WP and RP immune cell populations, a specific pattern of surface membrane receptors and a different expression of chemokines are needed [10].

In the last decades of the past century, researchers focused their attention on finding the basis of the immune system's innervation, revealing that primary and secondary lymphoid organs receive fibers from the autonomic nervous system [11–14]. In particular, a variety of experimental approaches has been developed to deepen our understanding of the nature of splenic innervation [13]. It has been widely described that sympathetic nervous system (SNS) fibers densely innervate the spleen [15], and their synaptic endings are in close contact with immune cells [16–18]. Released neurotransmitters reach the surface of macrophages and/or lymphocytes, interacting with their specific receptors and modulating immune cells responses (i.e., inhibiting or enhancing expression of different cytokines) [12,14,19]. These properties characterize the neuro-immune communication. Once activated, immune cells produce and secrete a variety of pro- or anti-inflammatory mediators, which provide a tuned immune cell response. Importantly, neuro-immune communications should not be considered a one-way pathway from neurons to immune cells. It has been described that a bi-directional communication does exist [20,21]. Not only neurotransmitters released by neuronal terminations influence immune cells activity, but, in turn, cytokines released from immune cells can influence

functions of neurons via cognate receptors on the neuronal cell surface [22–24]. Indeed, T cells are able to modulate noradrenaline (NA) pathways, reducing sympathetic fibers in the spleen and affecting the pituitary-adrenal axis in Central Nervous System (CNS) through diminished hypothalamic NA concentrations [24] (Figure 2).

In this view, the spleen appears as a crucial lymphoid organ, housing different populations of monocytes and macrophages, with B and T lymphocytes allowing both innate and adaptive immune responses. Besides this, the neuro-immune interaction provides a fundamental connection between the resident immune cells and the adjacent fibers of the autonomic nervous system innervating the spleen.
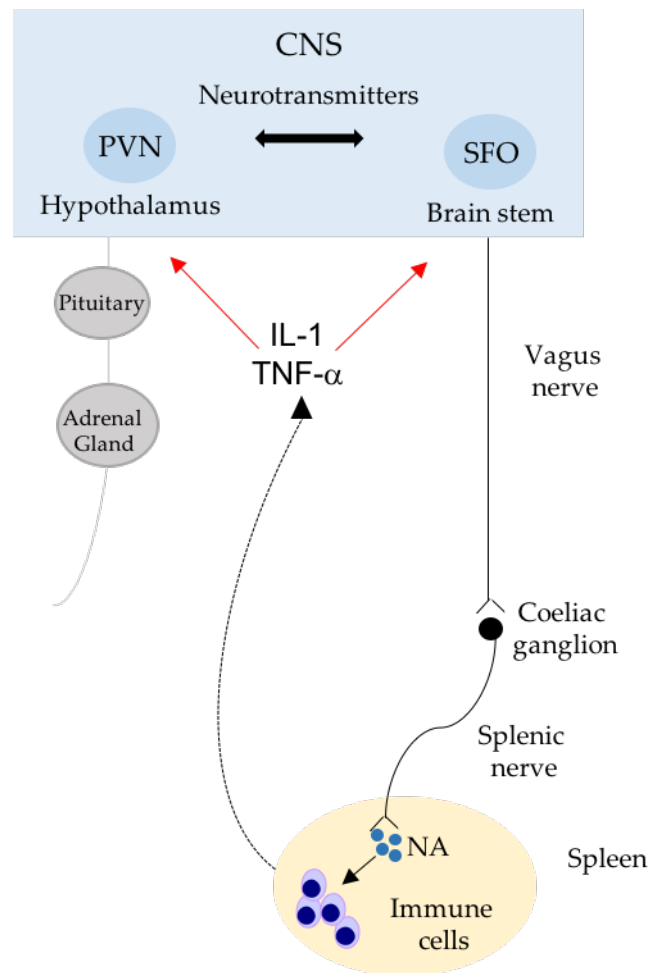
Figure 2. Bi-directional communication between the nervous and immune system. The scheme shows an integrative interface between the sympathetic nervous system (SNS), the hypothalamic-pituitary-adrenal (HPA) axis and the splenic immune reservoir. The SNS modulates immune cell activity through noradrenaline (NA) released in the spleen; and, in turn, the SNS activity is regulated by IL-1 and TNF-α cytokines, produced by splenic immune cells in a feedback loop manner. Paraventricular nucleus (PVN); subfornical organ (SFO); central nervous system (CNS). Double arrow: neuronal projections; red arrow: cytokine influence, black arrow: activation, dotted arrow: production.

2. Immune System and Metabolic Disease: Meta-Inflammation, Obesity and T2D, Adipokines

Obesity is a major health problem with growing incidence, especially in childhood, increasing the possibility of developing, in adulthood, metabolic or cardiovascular co-morbidities such as type II diabetes (T2D), atherosclerosis and hypertension [25–27]. Excessive nutrient intake and low energy consumption with a consequential increase in adipose tissue size are conditions that could lead to obesity. In parallel with the incidence of obesity, patients frequently develop insulin resistance (IR), a leading cause of type II diabetes (T2D). It is widely accepted that enhanced inflammation is a common

and central feature in metabolic syndrome. However, in contrast to the classic short-term inflammatory response to infectious agents that is typically followed by a return to baseline conditions, metabolic syndrome manifests alternative features of inflammation. A long-term and low-grade inflammation characterizes the so-called "meta-inflammation" [28]. Derangement in the immune cells homeostasis toward a steady increase in pro-inflammatory response is a key aspect that leads to the onset of such pathophysiological conditions [29].

Studies conducted both in humans and animal models outline that obesity-induced inflammation occurs in many organs involved in energy homeostasis: liver [30], muscle [31,32], pancreas [33], and adipose tissue [34]. Metabolic dysfunctions affect not only peripheral organs but also the central nervous system (CNS), especially in those hypothalamic areas involved in the control of energy balance, such as the arcuate nucleus (ARC), the ventromedial, lateral and paraventricular hypothalamic nuclei [35–37].

Despite all the biological aspects connecting obesity with related diseases that have not already been discovered, several studies have emphasized the relationship between obesity-induced pro-inflammatory response and the onset of metabolic syndrome. In order to investigate the causes of obesity-induced inflammation and IR, researchers at first focused their attention on fat pad. Lean adipose tissue is normally populated by different innate and adaptive immune cells, designated to maintain an anti-inflammatory phenotype and tissue integrity [38]. In addition, they have been shown to secrete anti-inflammatory cytokines with the aim of promoting insulin sensitivity, lipolysis, mitochondrial respiratory capacity, oxygen consumption and inhibiting lipogenesis [39].

Conversely, obesity provides conditions under which adipose tissue changes, altering the functions of immune cells, which contributes to the derangement of a balanced type-1 and type-2 immunity [29]. Importantly, murine models and human studies demonstrate that the quantity of adipose tissue macrophages (ATMs) bears a close relationship with the amount of adipose tissue itself.

Specifically, ATMs in lean mice account only for 10% of cells in the adipose tissue, and are evenly dispersed. In the lean state, macrophages express specific anti-inflammatory markers, such as MGL1 (macrophage galactose *N*-acetyl-galactosamine specific lectine 1), Arginase1, CD301, CD206 and CD163 on their cellular surface [40–42]; and the anti-inflammatory environment is maintained through the secretion of anti-inflammatory cytokines IL-4 (produced also by eosinophils), IL-13 and IL-10, with CD4+Foxp3+ regulatory T cells improving insulin sensitivity [29,40,42,43]. Since the controlled expression and secretion of anti-inflammatory mediators is pivotal to maintaining basal immune homeostasis in adipose tissue, ATMs and regulatory T cells are not the only cell types homing in on the adipose tissue of lean mice. Recently, a population of innate-like B, similar to B-1a and B10 B cells, has been identified in the visceral adipose tissue (VAT) of lean mice [44]. These cells, producing high amounts of IL-10, are critical players in maintaining the correct balance of immune cell populations toward an anti-inflammatory environment. The surgical procedure of splenectomy provided important evidence that the spleen is involved in the modulation of immune balance, being the source of innate-like B cells [44]. VAT of high fat diet (HFD)-fed splenectomized mice indeed showed reduced levels of innate-like B cells compared to sham mice, whereas the number of other lymphocytes was comparable [44]. In agreement, adoptive transfer of innate-like B cells in obese mice ameliorated both insulin sensitivity and glucose tolerance [44]. Others have discovered a different B cell population, called adipose natural regulatory B cells (Breg) [45]. This subset has been found to be crucial in decreasing CD44 and interferon-γ (IFN-γ) production in CD8+ T cells within visceral and subcutaneous fat depots. In addition, these cells were shown to improve IR by ameliorating tissue homeostasis through the constitutive production of IL-10, both in mouse models and in humans [45]. Through experiments of adoptive transfer of adipose tissue B cells in diet-induced obesity (DIO) mice, it has been demonstrated that Breg cells of non-splenic origin were found in adipose tissue, pointing out the possible presence of other routes of recruitment [44]. In a different work, it was shown that B cells are involved in the onset of inflammation and IR upon HFD feeding, promoting the release of CD4+ and CD8+ T cells cytokines and IgG2c pathogenic antibodies both in mouse models and in humans [46]. In addition, the IgGs produced enhanced the switch of macrophages toward pro-inflammatory phenotypes, thus contributing to development of metabolic disorders [46]. This

evidence highlights the fact that the surveillance of systemic immune homeostasis is granted by specific populations of B and T cells, which in part are provided by a secondary lymphoid organ, the spleen, that preserves an anti-inflammatory immune environment. On the other hand, the existence of B cell subpopulations that expand, boosting the early phase of inflammation in obesity, demonstrates that B cells are also involved in the first stage of metabolic syndrome with the production of autoantibodies.

In obesity, the immune scenario completely changes through the massive production and secretion of chemoattractant molecules or pro-inflammatory cytokines. Adipocytes progressively increase in number and dimension. Macrophages with a pro-inflammatory phenotype massively infiltrate the adipose tissue and, along with resident ATMs, begin pathological proliferative processes. The percentage of ATMs rises to 40–50%, and they are specifically detected surrounding the necrotic and enlarged adipocytes, forming typical "crown-like structures" (CLSs) [40,47–49]. ATMs confined in these structures are characterized by membrane expression of specific pro-inflammatory markers, such as CD11c, TLR4, and a reduced production of anti-inflammatory cytokine IL-10 [40]. In addition, ATMs (CD11c+CD206−) stimulated with pro-inflammatory cytokines such as IFN-γ, in turn produce abundant factors including TNF-α, IL-1β, IL-6 and nitric oxide (NO) [46,50]. A different study with HFD-fed mice showed that more pro-inflammatory F4/80+CD11c+ macrophages were recruited in adipose tissue of obese mice compared with lean mice and, conversely, HFD-fed C–C motif chemokine receptor 2 (CCR2) KO mice showed a decreased content of the same population of immune cells, thus suggesting that infiltrating cells were recruited through a mechanism mediated by the chemokine monocyte chemoattractant protein-1 (MCP1/CCL2) and CCR2 [38]. The progressive ATM accumulation in adipose tissue and the proportional increase in inflammatory mediator production give rise to a cascade of transcription factor activations and other pro-inflammatory cell recruitments, which dramatically and further strengthen the inflammatory response and progression of metabolic syndrome. Wu et al. demonstrated that, in obesity, the enrollment of splenic innate-like B cells, along with their anti-inflammatory activity, is not sufficient to counteract the rising inflammation and IR development [44]. With developing obesity, CD4+Foxp3+ regulatory T cells are no longer able to counteract pro-inflammatory IFN-γ secreting CD8+ and CD4+ T cells [28]. Natural killer (NK) cells in visceral adipose tissue are stimulated, in obesity, to produce IFN-γ, participating to the inflammatory response. Mice that were deficient of NK cells, adipocyte NK-activation receptors (NCR-1) or IFN-γ, demonstrated reduced recruitment of pro-inflammatory macrophages in VAT and ameliorated obesity-induced IR [51]. In contrast, IL-15-induced NK cells aggravated metabolic disease, favoring IR and adipose tissue inflammation [52]. As a further mechanism modulating the immune response involved in metabolic disease, it has been shown that adipose tissue releases free fatty acids (FFAs), which enhance inflammation trough Toll-like receptor 4 (TLR4) via Fetuin-A (FET-A) and the consequent activation of Nuclear factor KB (NF-KB) induced IR. In addition, it has been reported that expression of both FetA and TLR4 is essential for development of IR in the presence of FFAs [53].

Taken together, these findings suggest that the immune system plays a fundamental role in the maintenance of the homeostatic physiological environment. However, in response to specific stimuli, a dysregulation of the immune system may become a principal actor in the development of metabolic syndrome.

3. Metabolic Disease as a Risk Factor in CVD and Hypertension

In the contest of obesity, an abnormal increase of adipose tissue has been correlated with a concomitant increased risk of cardiovascular disease (CVD) incidence. Moreover, the contribution of different adipose depots in the development of obesity-related diseases has been investigated. Different studies on humans and mouse models, in fact, ascribe to VAT—and not to Subcutaneous Adipose Tissue (SAT)—a predominant role in IR and CVD [54–56].

As described above, adipose tissue can not only be considered as a place where energy is preserved but, notably, it also has endocrine activity producing a wide range of molecules involved in metabolic processes [37]. The proteins that exert immuno-metabolic or cardiovascular functions, and are produced and secreted by adipose tissue, are also known as adipokines. These molecules carry out

their effects modulating appetite and energy expenditure, insulin and glucose metabolism, as well as blood pressure, reaching the CNS and acting on neurons in specific brain areas. On this note, the melanocortin system resides in the hypothalamus and acts as a fulcrum of energy balance regulation [57]. Two different populations of neurons participate with opposite roles in the control of energy balance: on one hand, the orexigenic agouti-related peptide (AgRP) and neuropeptide (NPY) neurons; on the other, the anorexigenic proopiomelanocortin (POMC) neurons, whose activity can be directly regulated by metabolic hormones [57] and inflammatory molecules [35].

As a consequence of obesity, an immune regulator as important as IKKβ/NF-κB is involved not only in meta-inflammation of peripheral tissues, but is also activated in the CNS, participating in hypothalamic inflammation and aggravating IR and Leptin resistance development [35]. Leptin has been one of the first and most-analyzed adipokines. It is a hormone specifically expressed by adipose tissue, and its production is proportional to the quantity of fat pad [58]. The main role exerted by Leptin is the regulation of energy balance, acting directly in the CNS, and being able to cross the blood brain barrier (BBB) [59,60]. Besides its effect on energy control, Leptin is involved in the CNS modulation of sympathetic nerve activity (SNA). Targeted deletion of the Leptin receptor (ObR), specifically in the ARC, blocks diet-induced increased renal sympathetic activity and blood pressure rise [61]. Another study links Leptin action to the circumventricular subfornical organ (SFO), highlighting that deletion of Leptin receptors (ObR) in the SFO did not affect food intake behavior in mice, but suppressed SNA [62]. Studies conducted in both humans [63–66] and DIO mouse model clearly showed that, during obesity, the higher level of Leptin concentration correlated with SNA in renal nerves and blood pressure increase [67,68]. Thus, it is clear that obesity-related adipokines sustain neuroinflammation affecting many brain areas and producing different deleterious consequences to systemic homeostasis beyond feeding behavior and energy imbalance.

To date, many studies have described that the modulation of renal sympathetic activity is strictly related to hypertension induced by several pathophysiological alterations, like obesity [69]. However, it has recently been uncovered that sympathetic outflow plays a crucial role in hypertension, even directly modulating immune function though a control of splenic sympathetic nerve activity [12,13]. With obesity being characterized by chronic low-grade inflammation, and at same recognized as a dominant risk factor for hypertension, studies aimed at discerning the possible role of the neural modulation of splenic immunity in affecting the metabolic and cardiovascular consequences of the disease are warranted.

4. Neural Regulation of Immunity in Hypertension

The fact that immune cells and the sympathetic nervous system (SNS) strongly contribute to hypertension is a well-consolidated notion but, more recently, it has also been highlighted that these two elements are strictly intertwined in the modulation of blood pressure regulation. By using RAG-1$^{-/-}$ mice, lacking both B and T cells, it was firstly shown that lymphocytes, infiltrating tissues typically targeted by increased blood pressure levels, are essential for the onset of hypertension [70]. In the same experimental setting of Angiotensin (AngII)-induced hypertension, it has been demonstrated that the T cells found in target tissues as kidneys and vasculature originate in the spleen [12]. In particular, by taking advantage of mice differing for the leukocyte antigen CD45, expressed in the two isoforms CD45.1 and CD45.2 in two different strains, it was possible to generate chimeric animals by spleen transplantation in order to label T cells of splenic origin with the CD45.1 marker (CD45.1 donor mouse), differentiating them from those of the recipient mouse (CD45.2 positive). After infusion of AngII, T cells infiltrating aorta and kidneys were found to be positive for both CD45.1 and CD45.2 antigens, thus indicating that the hypertensive challenge recruited a movement of immune cells from the spleen to allow colonization of target organs [12]. However, whether an immune response originating in the spleen is also recruited in hypertension resulting from other causes, such as that reproduced with deoxycorticosterone acetate (DOCA)-salt or obesity murine models, is still unknown. Certainly, the recently discovered neural regulation of immunity during AngII-hypertensive challenge is suggestive that molecular mechanisms activated in the spleen to prime immunity could be shared by various stimuli capable of exerting a neurogenic action [12,13,71,72]. On this note, the fact that

chronic hypertensive stimuli, such as AngII and DOCA-salt, cause an increase of blood pressure levels activating renin-angiotensin system (RAS) in the brain through the AngII type 1 receptors (AT1Rs) in the SFO is well known [72]. This observation fits well with more recent evidence that both AngII and DOCA-salt activate an increased sympathetic outflow in the spleen [13], suggesting that, overall, different hypertensive challenges share neurogenic mechanisms to prime the immune system through neuroimmune pathways. Conversely, to date, there is still no evidence of the possible involvement of this neuroimmune pathway in the development of arterial hypertension associated with metabolic syndrome.

It is interesting to note that Harlan and colleagues, some years ago, demonstrated that the intracerebroventricular (ICV) administration of Leptin in the ARC of mice was able to significantly increase the renal sympathetic nerve activity, leading to arterial hypertension [61]. In light of current knowledge of neuroimmune pathways that are activated by sympathetic outflow in the spleen and are crucial for the onset of hypertension, it could be envisaged that even metabolic stimuli could share similar neurogenic mechanisms coupled with immune priming to increase blood pressure [62].

On a different note, immunologists discovered for the first time that the autonomic nervous system has a critical role in modulating immune responses in infectious settings. Several years ago, Tracey and colleagues published an amazing work that provided the basis for unraveling a circuit that connects the brain to the spleen and is able to dampen exacerbated innate immune responses during endotoxemia [17]. In particular, they showed that the electrical stimulation of the vagus nerve was effective for hampering the excessive TNF-α production induced by Lipopolysaccharide (LPS) administration in mice [17]. It was also proved that this pathway was mediated by α7-nicotinic cholinergic receptors (α7nAChRs) expressed on macrophages in the spleen, supporting the existence of the so-called "cholinergic anti-inflammatory reflex" [17].

Some other evidence, showed that the peripheral administration of the acetylcholinesterase inhibitor galantamine, typically used for the treatment of neurological disorders [73,74], has an anti-inflammatory effect, reducing serum TNF levels in endotoxemic mice [74]. Surprisingly, a similar galantamine treatment was shown to ameliorate inflammation and obesity in mice, significantly improving blood glucose and IR [75]. Indeed, mice fed with HFD and concomitantly treated with chronic galantamine exhibited a reduction of circulatory cytokines and adipokines, specifically IL-6 and Leptin [75]. Overall, these reports suggest that further insight is required to understand how neuroimmune mechanisms modulate metabolic, and probably cardiovascular, dysfunction in obesity.

Adipose tissue growth is closely related to angiogenesis [76]. Besides its role in secreting adipokines, regulating feeding behavior, and modulating SNA, Leptin plays an important role in angiogenesis, too [76]. During obesity, fat depots also produce other molecules involved in angiogenesis, such as the Vascular Endothelial Growth Factor (VEGF) [76], or the Placental Growth Factor (PlGF) [77], which belong to the family of the Vascular Endothelial Growth Factors (VEGFs). These growth factors have been considered novel therapeutic targets for the treatment of human obesity, given results obtained in experimental models suggesting the protective roles of antiangiogenic molecules, highlighting their potential therapeutic application [76]. On a similar note, the more recent role proposed for PlGF as a modulator of inflammatory and immune responses in cardiovascular settings further suggests that its involvement in metabolic syndrome could deserve further investigation, being involved in roles other than angiogenesis [78–80]. Indeed, the fact that PlGF has also been shown to play a pivotal role in the onset of hypertension, by mediating neuro-immune interactions in the spleen [12], suggests the possibility that its implications in obesity could also be related to the cardiovascular consequences of metabolic alterations, as is the case for increased risk of hypertension itself. A report in the literature suggests that the inactivation of PlGF in a murine model of HFD-induced obesity leads to reduced adipose tissue growth with a concomitant reduction of angiogenesis [77]. In *ob/ob* mice, a murine model of obesity characterized by mutations in the gene responsible for the production of Leptin, the absence of PlGF significantly prevented weight gain [81]. However, as mentioned above, obesity is also strictly related to chronic inflammation and increased risk of developing cardiovascular disease [47,48], thus suggesting the need to explore possible roles of

angiogenic growth factors in mediating the immune functions involved in obesity and related complications.

5. Conclusions

Obesity and related metabolic disorders are recognized as risk factors for the onset of cardiovascular diseases, and hypertension in particular. Both these conditions share complex and not-yet-completely-understood aspects. The immune system is not only the main player responsible for preserving the host from exogenous hostile infiltration, but increasing evidence sustains the idea that it is also involved in the regulation of multiple biological aspects. Derangements in the homeostasis of immune components are pivotal factors in the onset of metabolic diseases and end-organ damage in hypertension. Despite this, the origin and the activation pathways of infiltrating immune cells in a multifactorial disease, such as metabolic syndrome, have not been fully described yet. However, impaired activity of the autonomic nervous system has been deepened in response to several metabolites produced during obesity and insulin resistance. The recent discovery of a neuro-immune interaction that primes activation and migration of splenic immune cells has revealed that the nervous system modulates immunological responses evoked by hypertensive stimuli. Therefore, it can be hypothesized that the same neuro-immune communication in this secondary lymphoid organ contributes to the refueling of pathological immune cells in adipose tissue during metabolic disease.

Conflicts of Interest: The authors declare no conflict of interest.

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Moreover, during my PhD, I contributed to the realization of a project whose article is now "in press" on *Cardiovascular research*.

Title: "DOCA-salt hypertension activates PlGF in the spleen to couple sympathetic drive and immune system activation".

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8. Acknowledgments.