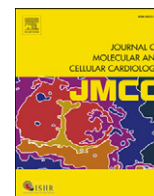


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Review article

Toll-like receptor-4 signaling pathway in aorta aging and diseases: “its double nature”

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ABSTRACT

Recent advances in the field of innate immunity have revealed a complex role of innate immune signaling pathways in both tissue homeostasis and disease. Among them, the Toll-like receptor 4 (TLR-4) pathways has been linked to various pathophysiological conditions, such as cardiovascular diseases (CVDs). This has been interrogated by developing multiple laboratory tools that have shown in animal models and clinical conditions, the involvement of the TLR-4 signaling pathway in the pathophysiology of different CVDs, such as atherosclerosis, ischemic heart disease, heart failure, ischemia-reperfusion injury and aorta aneurysm. Among these, aorta aneurysm, a very complex pathological condition with uncertain etiology and fatal complications (i.e. dissection and rupture), has been associated with the occurrence of high risk cardiovascular conditions, including thrombosis and embolism. In this review, we discuss the possible role of TLR-4 signaling pathway in the development of aorta aneurysm, considering the emerging evidence from ongoing investigations. Our message is that emphasizing the role of TLR-4 signaling pathway in aorta aneurysm may serve as a starting point for future studies, leading to a better understanding of the pathophysiological basis and perhaps the effective treatment of this difficult human disease.

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1. Introduction

In the Western population, the incidence of cardiovascular diseases (CVDs) has increased due to ongoing aging. This represents a health, economic and social problem mainly due to the dramatic increase in the number of non-autonomous affected individuals. In addition, it is estimated that the CVD incidence will reach 36.9% to 40.5% by 2030, without changes in prevention or treatments [1]. Thus, CVDs are a very challenge. However, progress has been made in recent years for the treatments of some CVDs, such as ischemic heart diseases, due to new thrombolysis and percutaneous coronary intervention procedures [2]. Conversely, for a large CVD group, the management and the outcome still remain difficult. Typical examples are the aneurysms, and in particular, sporadic thoracic aortic aneurysms (TAA), whose incidence is increasing in our population, and especially in elderly [3,4]. The etiology of TAA is heterogeneous and can be classified in syndromic and non-syndromic forms. The first includes inherited and familial TAA that characterize younger patients, the second group includes sporadic or isolated TAA, that occur in advantage age. Inherited TAAs have an incidence of <5% and are represented by Marfan syndrome, Ehlers-Danlos syndrome, Loeys-Dietz and aneurysm-osteoarthritis syndrome. Familial TAAs have a 20% incidence and are represented by TAA associated with bicuspid aortic valve, patent ductus arteriosus (PDA) and cerebrovascular disease. Isolated or sporadic TAAs have a degenerative etiology linked to classic cardiovascular risk factors, such as smoking, hypertension, and hyperlipidemia. Table 1 provides an overview of syndromic and non-syndromic TAAs, with their corresponding clinical features [3,4].

The detection of TAA is often fortuitous, occurring during a routine physical examination or an independent medical evaluation [3,4]. Once suspected, the diagnosis must be confirmed by imaging clinical modalities (i.e. X-ray, magnetic resonance imaging, computed tomography scanning, or ultrasound), which allow the selection of the surgery

Table 1
Clinical features of thoracic aortic aneurysm.

Disorder	Main CV features	Additional clinical features
Marfan syndrome	Aortic root aneurysm, aortic dissection, mitral valve prolapse, main pulmonary artery dilatation, left ventricular dysfunction	Lens luxation, pectus deformity scoliosis, flat feet, increased arm span
Ehlers-Danlos syndrome	Arterial rupture and dissection, severe valvular insufficiency	Translucent skin, dystrophic scars, thin lips, deep set eyes
Loeys-Dietz syndrome	Aortic root aneurysm, aortic dissection, arterial valve prolapse, congenital cardiac malformations	Bifid uvula/cleft palate, hypertension scoliosis, club feet, pectus deformity
Aneurysm-Osteoarthritis syndrome	Aortic root aneurysm, aortic dissection, arterial aneurysms and dissections, arterial tortuosity, mitral valve prolapse, congenital cardiac malformations	Osteoarthritis, soft skin, flat feet, scoliosis, recurrent hernia, hypertelorism pectus deformity
Arterial tortuosity syndrome	Arterial tortuosity, arterial stenosis and aneurysm	Hyperlax skin and joints
Cutis laxa syndrome	Aortic root aneurysm, arterial tortuosity	Hyperlax skin and joints, emphysema
Familial thoracic aortic aneurysm syndrome	Thoracic aortic aneurysm, dissection	None
Familial thoracic aortic aneurysm syndrome with bicuspid aortic valve	Calcified aortic valve	None
Familial thoracic aortic aneurysm syndrome with PDA	PDA	None

PDA, Patent ductus arteriosus.

procedures (including elective surgery or endovascular repair), before the onset of catastrophic and life-threatening complications (i.e. dissection or rupture) [3,4]. Furthermore, there are no available biomarkers at this time for early diagnosis of sporadic TAAs, although emerging evidence points out some of the tissue and serum molecules, which might play this putative role (see Table 2). On the other hand, sporadic TAAs have been the object of a very small number of investigations than familial forms [4]. The molecular and genetics mechanisms of then on-familial TAA forms (see Table 1), which represent the major number of cases of TAAs, remain largely unknown [3,4]. As a result, it is difficult to generalize on disease pathways or genetic risk factors that contribute to sporadic TAA. In fact, this disease is considered to be a pathology by unclear mechanisms, and with a very complex clinical presentation, characterized by the lack of overt symptoms, until dissection or rupture occur [5,6]. As result, better characterization at the molecular level of sporadic TAAD is necessary.

We recently summarized, for the first time, the very limited literature data on genetic studies of sporadic TAA in order to identify pathways of disease and their genetic variants that modulate its susceptibility [4]. In Chen, Madonna, Milewicz and colleagues, the role of genetic predisposition in the genesis of TAAs has been defined, and in particular it has been shown that loss of smooth muscle α -action leads to NF- κ B-dependent increased susceptibility to Angiotensin II (Ang II) in smooth muscle cells and aortic enlargement [7]. In addition, we postulated a pattern of TAA onset, already defined by Ruvolo, Balistreri and colleagues [8] as *double-face signal pathway model*, given its features (see Fig. 4 of Ruvolo et al., 2014 study [8]; <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4120489/>). The model is centered on an innate immunity receptor signaling pathway of [9], the *Toll-like receptor-4 (TLR-4) signaling pathway*, which has the role of a hub. TLR-4 is recognized to have a key function in the host defence against Gram-negative bacteria, viruses, fungi and mycoplasma [10], but also in the patho-physiology of several age-related diseases [9,11], including CVDs, as stressed in our studies [4,8,11,12] and described by other groups [13–15]. Recently, its role has emerged in maintaining aortic homeostasis, but also in establishing aorta aneurysm [4,8,16–29].

Based on these observations, in this report we will stress the functional importance of TLR-4 signaling pathway both in maintaining aortic homeostasis, and evoking aorta aging and disease, such as sporadic TAA, which will be object of intense description and discussion.

2. Structural and functional features of the aorta in physiological conditions and during aging

For a better understanding of all the concepts, that will be reported and discussed, we will briefly describe the aorta structure and its changes during aging. Subsequently, we will emphasize the molecular and cellular mechanisms associated with aorta aging.

The aorta is the largest blood vessel in the human body and originates from the left ventricle of the heart, from which it carries oxygenated blood throughout the body. The aortic size is proportional to the height and weight of an individual and can be divided into several sections: ascending aorta, descending aorta and aortic arch, which includes the thoracic and abdominal aorta (see Fig. 1). The aortic wall consists of three layers (tunica intima, media and adventitia), which have an anatomical structure similar to that of other vessels, and which play different roles in aorta development and homeostasis, and yet in its pathologic degradation. In particular, the tunica media consists of concentric bands of elastin, collagen, and vascular smooth muscle cells (VSMCs), so called lamellar units. It provides visco-elasticity and it is the site of degenerative remodeling responsible for aneurysm formation (see Fig. 2) [30].

Various sections of aorta are also characterized by a different embryonic origin. In particular, the aorta section ranging from the ascending and aortic arch to the arterial ligamentum derives from precursor cells of the neural crest, while abdominal aortic section arises from

Table 2
Putative tissue and serum biomarkers of thoracic aortic aneurysm.

Name	Molecular function	Clinical findings	Proposed clinical applications	Clinical implementation	Ref.
Kallistatin	Protease inhibitor	↓ in postsurgical sera	Postsurgical outcome	No	[159]
α-2-macroglobulin	Acute-phase protein, signaling molecule	↓ in postsurgical sera	Postsurgical outcome	No	[159]
D-dimer	Fibrin fragment	↑ dissected TAA	Rule out TAA dissection	No	[160]
hs-TnI	Cardiac sarcomeric protein	↑ dissected TAA	Rule out TAA dissection	Yes	[160]
TnI	Cardiac sarcomeric protein	↑ dissected TAA	Rule out TAA dissection	Yes	[161]
NTproBNP	Cardiac natriuretic peptide	↑ dissected TAA	Rule out TAA dissection	No	[162,163]
RCP	Acute-phase protein	↑ in non-surviving patients with dissected TAA	Prognostic factor of in-hospital mortality after TAA dissection	No	[164]
Hypermethylated ACTA2	Aortic contractile protein	↑ dissected TAA	Risk factor of TAA development in the bicuspid population	No	[7,165]
Hypermethylated PTPN22	T cell receptor signaling	↑ dissected TAA	Risk factor of TAA development in the bicuspid population	No	[165]
Hypermethylated TGFβ1	Multifunctional protein that controls proliferation and differentiation	↑ dissected TAA	Risk factor of TAA development in general population	No	[165]
TN-C		↑ dissected TAA undergoing regression	Predictive factor of dissected TAA regression	No	[166]
IL-6	Extracellular matrix glycoprotein	↑ dissected TAA	Risk factor of TAA development in the general population	No	[167]
TNF-α	Acute-phase proinflammatory protein	↑ dissected TAA	Risk factor of TAA development in the general population	No	[168]

TAA, thoracic aortic aneurysm; TnI, troponin I; hs-TnI, high sensitive troponin I; NTproBNP, N-terminal pro-brain natriuretic peptide; RCP, reactive C protein; ACTA2, Actin, Alpha 2, Smooth Muscle, Aorta; PTPN22, Protein Tyrosine Phosphatase, Non-Receptor Type 22; TGF, tumour growth factor; TN-C, Tenascin C; IL-6, interleukin 6; TNFα, tumour necrosis factor alpha.

mesodermic precursor cells [31]. These sections show a tunica media that grows through sequential assemblage of lamellar units, reaching a total of 55–60 units in adulthood. This allows of maintaining a constant ratio between aortic diameter and medial thickness. However, the thickness of each unit is expanded during maturation. The heterogeneity of various aortic traits and segmental growth patterns reflects the differences in the density of extracellular matrix (ECM) microfibril and VSMC reactivity to vasoactive growth factors, with downstream effects

linked to activation of different pathways [4,31], which are different and able to determine a different susceptibility to the onset of aneurysm [32].

In addition, the aorta, as well as the entire cardiovascular system (heart and vascular system) and any other tissue, organ or system, exhibit several age-related changes, both at anatomical and physiological levels, as clearly demonstrated by Collins and colleagues [30]. Changes in the aortic structure occur throughout life, but they are mostly

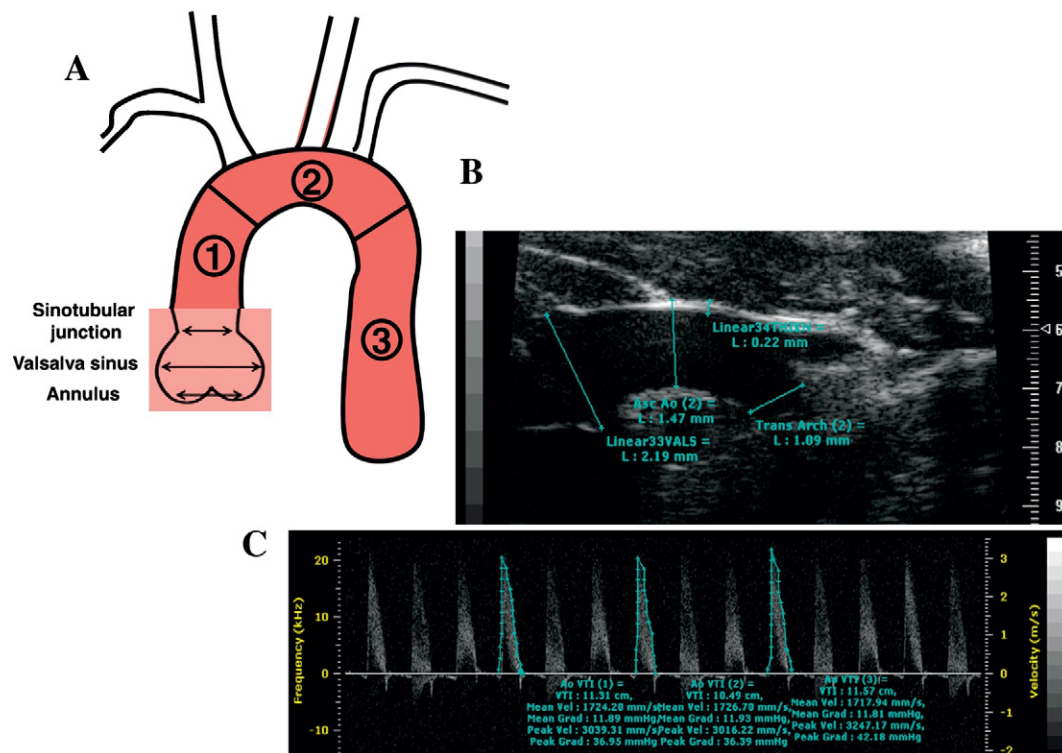


Fig. 1. Anatomy of the thoracic aorta. Panel A: schematic representation of the thoracic aorta, which is divided into several segments: ascending aorta (1); aortic arch (2); descending aorta (3). Panel B: representative two-dimensional echocardiogram image of the thoracic aorta from C57 mouse in parasternal long-axis view. Panel C: representative aortic pulse Doppler profile of the thoracic aorta from C57 mouse (original images from personal contribution). Two-dimensional and echocardiography images and doppler signals were recorded and analyzed using a Vevo 770 equipped with a 40 MHz ultrasonic linear probe (Visual Sonics Inc., Toronto, Ontario, Canada). Images were obtained in the parasternal long-axis view. Aortic diameter measurements were made in late diastole.

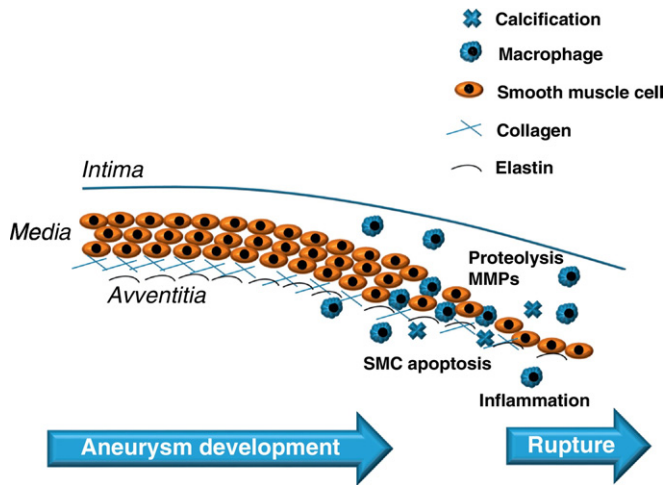


Fig. 2. Schematic representation of thoracic aortic aneurysm (TAA) pathophysiology. SMC, smooth muscle cells; MMPs, metalloproteinases.

symptomatic in middle age individuals. Typical age-associated aorta phenotypes are the vascular remodeling (VR) and the medial degeneration (MD) (see Fig. 3) [31–40]. Endothelial dysfunction, increased oxidative stress, inflammatory reaction, inflammatory cell infiltration in the aortic wall, apoptosis of VSMCs, degeneration of aortic media, and elastin fragmentation and degradation represent their microscopic alterations (see Fig. 3) [32–38]. These changes are responsible for the decline in aorta's elasticity [32–38]. As a result, the aorta dilates and mechanical expansion forces are transferred to ECM elements within the aortic wall, and in particular to collagen [32–38]. In turn, this causes collagen remodeling within the tunica media, resulting in a stiffer and less compliant vessel. Increased aortic stiffness causes increased systolic and peak pressures, as well as an increase in workload on the heart [32–38].

Various symptoms and illnesses have been associated with age-related aortic VR and MD, including hypertension, atherosclerosis (angina,

arrhythmia, congestive heart failure, coronary artery disease, stenosis, general ischemia), orthostatic hypotension, aortic aneurysm and dissection [38]. All these pathologies show an increased incidence in our populations and particularly in old people [38].

3. Molecular and cellular mechanisms of aorta aging: the role of “sterile inflammation”

There is a close relationship between aging and CVDs, as recently pointed by North and Sinclair [39]. Aging itself is not a disease. However, it significantly increases the risk of chronic inflammatory-related diseases, including CVDs, as outlined above [9,11, and 40]. The most plausible relationship is related to the intrinsic characteristics of aging, which causes many forms of damages at the molecular, cellular, and tissue level [41]. In turn, this results in a reduced body response and function [41]. Specifically, during aging the cells exhibit different abnormalities, including genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and modification of intercellular communication leading to cardiovascular dysfunction (see Fig. 4) [41–43]. This tissue damage causes cell death and chronic inflammation, so-called “sterile inflammation” (indicating absence of detectable pathogens) or *inflamm-aging* [42,43] through the release of the so-called *senescence associated secretory phenotype* (SASP) [44–46]. SASP is represented by a myriad of factors collectively called *damage-associated molecular patterns* (DAMPs) [40,44–48]. This condition is supported by “danger theory” introduced by Polly Matzinger [49], based on the concept that the primary function of the immune system stays in detecting and protecting the host against danger. Thus, the initiation of the immune response is not represented by foreign or stranger microbes (non-self), but by alarm signals generated by injured or damaged cells and tissues, collectively referred to as DAMPs [46–48].

DAMPs induce sterile inflammation through their binding to innate immune receptors, collectively defined as pathogen recognition receptors (PRRs), including advanced glycosylation end product-specific receptor (AGER/RAGE), Toll-like receptors (TLRs), NOD1-like receptors (NLRs), RIG-I-like receptors (RLRs), and AIM2-like receptors (ALRs)

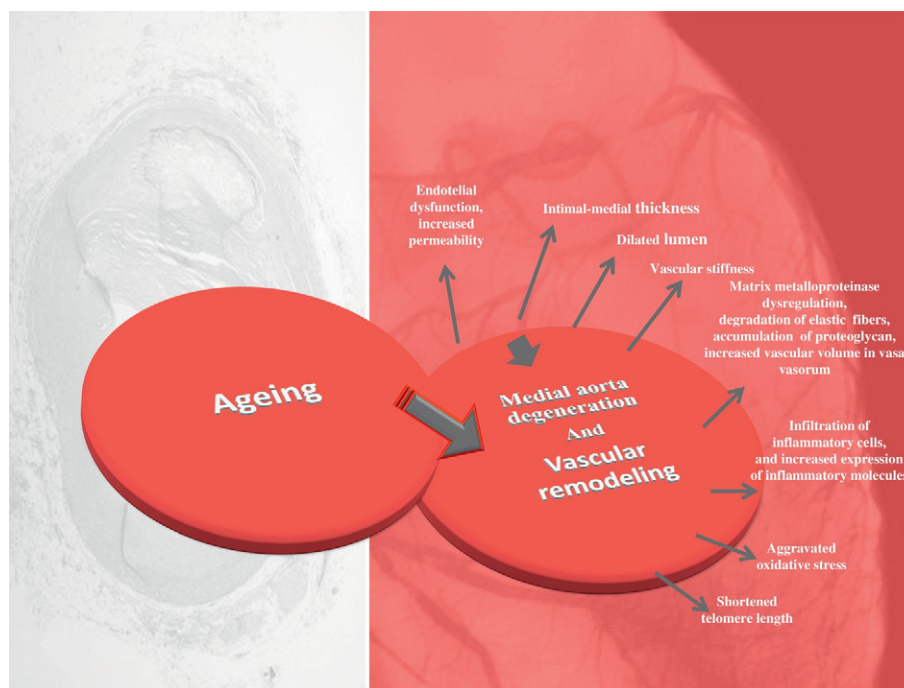


Fig. 3. Vascular aging and typical aorta aging phenotypes (medial degeneration and vascular remodeling). Vascular aging determines the onset of medial degeneration and vascular remodeling, which are associated with several changes at microscopical level, including endothelial dysfunction, increased oxidative stress, inflammatory reaction, inflammatory cell infiltration in aortic wall, shortened telomere length, apoptosis of VSMCs, degeneration of aortic media, and elastin fragmentation and degradation, represent their microscopic alterations.

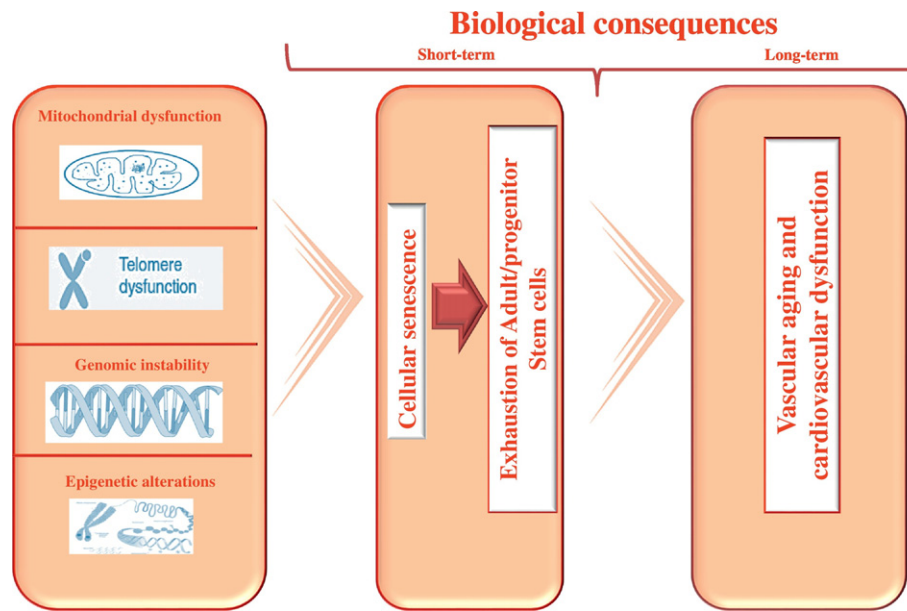


Fig. 4. Intrinsic changes associated with vascular aging and cardiovascular dysfunction. Several intrinsic changes, including mitochondrial dysfunction, telomere attrition and dysfunction, genome instability and epigenetic alterations, induce severe biological consequences. Precisely, at short-term, cellular senescence of cells of cardiovascular system and exhaustion of stem/progenitor cells involved in cardiovascular repair, are evoked. As a result of the long-term effects, they, in turn, determine vascular aging and the consequent cardiovascular dysfunction, responsible of the development of several CVDs, such as sporadic TAA.

[50]. The evocation of sterile inflammation causes tissue inflammatory/immune cell infiltration, and thus it acts as vicious cycle to further induce tissue and cell damages, and DAMP release. All factors will determine the onset of age-related diseases [41,43, and 47].

In an attempt to suppressor delay age-associated aorta VR (i.e. aneurysm), VR has been studied in various species including rats, rabbits, nonhuman primates, and humans, being an evolutionarily conserved process (see Table 3). The findings obtained have provided insights into the molecular and cellular mechanisms of aorta aging in humans [51–60]. Precisely, it has been shown that age-associated aorta VR is the result of a sterile inflammation, probably mediated by two supposed mechanisms: 1) infiltration of immune cells, that degrade tissues and release reactive or toxic molecules, causing DAMPs production [61]; 2) phenotypic changes in endothelial cells (ECs) and VSMCs evoked by altered and overload expression of stress and stretch signaling

pathways, triggered by different stressors or damage tissue stimuli throughout life (i.e. hypertension, aging and smoking) [34,36,37,61, 62]. In particular, rennin/AngII, aldosterone/mineral corticoid receptor (Aldo/MR) and the endothelin-1 (ET-1)/endothelin-1 receptor A (ETA) signaling system cascades seem to act by inflammatory drivers. This evidence is the result of findings from the Lakatta s' group, using rat models, [62,63], and Šabovic's group [64], who propose administering combination of low-dose fluvastatin and valsartan, as anti-aging drugs. On the other hand, the expression and activity of Ang II, the angiotensin converting enzyme (ACE), the Ang II receptor AT1 receptor within the aorta wall, in particular, in the thickened intima in several species, including humans, are risen as the age progress [62–71]. Interestingly, also the chymase, another angiotensin-convertase, was found in the wall of aged aorta [69]. Recently, two additional groups [70,71] evidenced that Ang II signaling cascades promote aorta remodeling by

Table 3
A summary of studies on age-associated vascular remodeling.

Studies (years)	Topic	Studied model	Findings
Wang M. et al. [51]	Angiotensin II in age-associated carotid arterial remodeling	Rats	Angiotensin II activates MMP 2 and mimic age-associated carotid arterial remodeling
Jiang L. et al. [52]	Aortic Calpain-1 activity and aging in vascular smooth muscle cells	Rats	Increased aortic calpain-1 activity mediates age-associated angiotensin II signaling of vascular smooth muscle cells
Wang M. et al. [53]	MMP 2 and angiotensin II in aging	Nonhuman primates	Aging increases aortic MMP 2 and angiotensin II activity in nonhuman primates.
Wang M. et al. [54]	Aging of grossly human aortic wall	Humans	Proinflammatory profile within the grossly normal aged human aortic wall
Wang M. et al. [55]	MMP2 and aortic remodeling during aging	Rats	Increased MMP2 activity and aortic remodeling during aging
Wang M. et al. [56]	MMP 2 and TGF-beta 1 signaling within aged arterial wall	Rats	MMP 2 activate TGF-beta1 and TGF-beta 1-type II receptor signaling in aged arterial wall
Spinetti G. et al. [57]	MCP-1 and CCR2 signaling in smooth muscle cells during aging	Rats	Aortic MCP-1 and its receptor CCR2 increase with age and alter vascular smooth muscle cell function
Asai K. et al. [58]	Vascular endothelial dysfunction and apoptosis during aging	Monkeys	Vascular endothelial dysfunction was present in oldmonkeys without evidence of atherosclerosis, which may be due to endothelial apoptosis and reduced endothelial cell density
Wang M. et al. [59]	Proinflammatory signaling in age-associated arterial remodeling	Rats	A local proinflammatory signaling loop facilitates adverse age-associated arterial remodeling
Song Y. et al. [60]	Expression of proinflammatory mediators in vascular smooth muscle cells during aging	Mice	Aging enhances gene and protein expression of IL-6, CCL2 and TLR4 in vascular smooth muscle cells

MMP2, metalloproteinase type 2; TGF-beta, tumour growth factor beta; MCP-1, Monocyte chemoattractant protein-1; CCR2, chemokine receptor type 2; IL-6, interleukin 6; TLR4, Toll-like receptor 4.

inducing expression and/or activity of several pro-inflammatory transcription factors (located downstream to these pathways). Among these, first is the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) [41,61,71]. Its activation determines the production and release of a myriad of factors, including inflammatory mediators, mitotic and tropic factors, proteoglycans and metalloproteinase (MMP)s (especially MMP-2 and -9), and vaso-active molecules. Overall, they constitute the so-called *age-associated arterial secretory phenotype* (AAASP) (see Fig. 4 of Ruvolo et al., 2014 study [8]; <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4120489/>) and create a microenvironment, which drives the phenotype shift of both ECs and VSMCs. Thus, ECs and VSMCs become secretory, migratory, proliferative and senescent, resulting in changes in aorta remodeling. These last include intimal-medial thickening, fibrosis, calcification, and aneurysms, associated with reduction in endothelial-dependent vasodilatation and increased stiffness. Among AAASP components, the main contributors to aorta aging and remodeling are MMP-9 and -2, which degrade collagen, elastin, and other ECM molecules, resulting in increased DAMPs [72,73]. Their relevance in vascular aging has been confirmed by MMP inhibition studies. In particular, Lakatta's group used chronic administration of broad-spectrum MMP inhibitor, PD166739 in 16-month-old rats for 8 months. The results have shown that effective MMP inhibition delays age-associated arterial proinflammatory signaling, and this is accompanied by the preservation of intact elastin fibers, collagen reduction in, and the decrease of age-associated increase in blood pressure [74]. Thus, MMP activation plays a central role in aorta age-related remodeling and consequently in pro-inflammatory aorta remodeling and stiffening, which are typical pathological entities of several aorta diseases. These include hypertension, atherosclerosis and aneurysm/dissection, showing regional specific onset associated with regional aorta embryologic origin, regional disease susceptibility and genetic factors associated with diseases [4,35,75].

4. Toll-like receptor-4 (TLR-4) signaling pathway in aorta homeostasis and health

Previous evidence has shown a key role of TLR-4 signaling pathway in the onset of myocardial diseases (i.e. septical cardiomyopathy, ischemia/reperfusion, heart failure, cardiac hypertrophy and toxic cardiomyopathy) [13–15]. In particular, it has been supposed its role of hub in mediating a tight relationship between induction of chronic inflammation and disease development [9,11]. This is consistent with its expression not only in immune cells, but also in a wide range of tissue cells, such as those of the cardiovascular system, and its ability to active immune/inflammatory responses by binding exogenous and endogenous ligands (see their description below) [9,11]. On the other hand, a common feature of the pathophysiology of a large number of CVDs, including myocardial diseases, is the involvement of a robust inflammatory response [9,11]. However, a recent meta-analysis of 15,148 subjects demonstrated the absence of significant associations of the two *TLR-4* gene (MIM: 603030) variants [+896A/G (Asp299Gly; rs4986790) and +1196C/T (Thr399Ile; rs4986791)] with susceptibility to myocardial diseases, although other studies emphasize strong associations [76]. According to these findings, our data showed significant opposite role of rs4986790 TLR-4 variant in longevity and myocardial infarction, as underlined in the study published in 2004 [77] and summarized in our reviews [9,11,78,79]. In fact, we concluded that the rs4986790TLR-4 polymorphism, which attenuates receptor signaling, increases the risk of infections and decreases the risk of atherogenesis, presumably limiting inflammatory responses. Thus, rs4986790 variant could have a higher probability of longevity in a modern environment with reduced pathogenic load and better control of severe infections by antibiotics [79].

In fact, the role of TLR-4 mediated receptor signaling pathway in the context of aorta diseases is also emerging [16–29]. In particular, our group postulates its fundamental contribution to the development of

sporadic TAA, as reported in the above mentioned model [4,8]. In this report, we will discuss these aspects with particular emphasis, considering the growing evidence of current investigations. Revealing the role of TLR-4 signaling pathway in sporadic TAA may serve as a starting point for future studies that lead to a better understanding of the pathophysiological basis and perhaps effective treatment of this human disease.

In the following paragraphs, the TLR-4 pathway will be pointed, including the function, expression (the most well-know TLR members), and its modulation induced by genetic variants. In addition, experimental evidence on the involvement of TLR-4 pathway in aorta health and aorta aneurysms is also described, in particular discussing potential and hypothetical models on its role in sporadic TAA pathogenesis.

4.1. TLR-4 pathway: structure, ligands, functions, cell expression and modulation by genetic variants

The TLR-4 pathway, identified as the first human homologue of the *Drosophila* Toll [80,81], consists of three domains: an extracellular leucine-rich repeat (LRR) domain, a trans-membrane domain, and an intracellular Toll-interleukin-1 receptor (TIR) domain. The extracellular LRR domain is involved in the recognition of the lipopolysaccharide (LPS) of Gram-negative bacteria, the prototypic TLR-4 ligand or specific Pathogen Associated Molecular Patterns (PAMPs). Other exogenous TLR-4 PAMPs are the fusion protein of respiratory syncytial virus, the envelope protein of mouse mammary tumour virus, and others as summarized in a recent review from Mukherjee and colleagues [10]. Furthermore, endogenous molecules produced by cell and tissue damage, DAMPs, can interact directly or indirectly with the TLR-4 pathway, such as heat-shock proteins (HSPs), hyaluronic acid, β -defensin-2, oxidized-LDL (ox-LDL), fibronectin, heparin sulfate and fibrinogen, and amyloid peptide and others [47]. Its activation involves a downstream signaling mediated by several intracellular adaptor molecules, inducing the activation of various transcription factors, such as NF- κ B, and consequently the release of several inflammatory mediators [11]. The TLR-4 pathway also triggers the instructive immunity. In antigen-presenting cells, TLR-4 pathway activation induces the expression of costimulatory molecules and the Major histocompatibility complex class II antigens, which help to support the activation of instructive responses [11]. TLR-4 is actually expressed on several immune cells, including classic antigen-presenting cells (dendritic cells, monocyte-macrophage cells), as well as B and T cells. These data confirm the capacity of these receptors to link innate and instructive immunity [11]. Different types of tissue cells can also respond to inflammatory stimuli through TLR-4, which is also expressed in epithelial cells at potential sites of pathogen entry, including skin, respiratory, intestinal and genitourinary tracts, and on EC and VSMC cells, but also in other tissue cells [11].

The activity and function of TLR-4 seem to be modulated by genetic variations, mainly by single nucleotide polymorphisms (SNPs). The gene sequencing (MIM: 603030; mapping in human beings in the 9q32-q33 region) of several mammals has identified several genetic variants mainly in the LRR domain [11]. This suggests a high conservation of innate pathways among organisms that involves the TIR domain. In any case, this concept seems to be in line with the finding that the innate immune system shows a high degree of homology in different organisms, including mammals, insects, and plants, and apparently it has been strongly preserved during evolution [11]. Accordingly, the strong variation of the LRR region, involved in the recognition of PAMPs (or DAMPs), is probably the result of evolutionary pressure induced by host's pathogens [11]. In addition, most of these variants are in the third exon, that encodes the LRR region, and they have a low frequency in human populations (<1%) [11]. On the other hand, in humans a higher frequency (>5%) has been evidenced for only two SNPs, +896A/G (Asp299Gly; rs4986790) and +1196C/T (Thr399Ile; rs4986791) (above mentioned). The two SNPs, as first suggested by Arbour and colleagues [82], induce a blunted response to LPS. In addition, it has been described that the two SNPs exist in a co-segregated (Asp299Gly/

Thr399Ile) state [82]. The co-segregation of the two SNPs implies four haplotypes, referred to as wt/wt, Asp299Gly/wt, Thr399Ile/wt, and Asp299Gly/Thr399Ile [82]. They are differently represented in the world population with a specific geographic distribution. This might be the result of differences in environmental pathogenic pressure [83,84] in the human population during the migration into Europe, Asia, and the New World [85,86]. In addition, these TLR-4 haplotypes are phenotypically associated with changes in the production of cytokines, mainly those carrying the Asp299Gly mutation [83–86]. In particular, the Asp299Gly/Thr399Ile haplotype seems to induce a phenotypic effect characterized by reduced production of pro-inflammatory cytokines [85,86]. On the other hand, conflicting data have emerged on the phenotypic effect of the four TLR-4 haplotypes. Discordant results also exist in the literature on the role of the two TLR-4 SNPs not only for the risk of myocardial diseases (as above described), but also for other age-related diseases, such as Alzheimer diseases, prostate cancer, and longevity, as also described in our studies [9,11,76–79].

4.2. Experimental evidence on the involvement of TLR-4 in aorta health and disease

As above reported, the TLR-4 signaling pathway is also expressed in EC and VSMC cells. As a result, in 2008 Pryshchep and her group [87] examined the expression profile of TLRs from 1 to –9 in six different human macrovessels (temporal, carotid, subclavian, mesenteric, iliac, and thoracic aorta) collected during postmortem examinations of 37 donors (mean age 64 years; 25 males, 12 females; dead for causes unrelated to aortic disease and without sepsis at death time, as confirmed by autopsy), by using quantitative-RT-PCR and immuno-histochemistry with primary antibodies (CD11c, TLR2, TLR-4, TLR5, CD86). Their results demonstrated that the TLR expression levels were significantly different ($p < 0.001$), by comparing the 6 vascular territories. In particular, the data obtained demonstrated a ubiquitous and abundant expression of TLR-4 among the 6 macro-vessels, compared to the other TLRs. However, higher TLR-4 levels were detected in the three aorta laminae. This heterogeneity has led Pryshchep and her group to suggest the existence of a functional specialization in the artery surveillance, with each vascular lamina region dedicated to a selected spectrum of TLR-4 ligands. In addition, they propose that this specialization of blood vessel territories may reflect the stringent target tissue tropism of target tissue of inflammatory vasculopathies [87].

In a specific aortic case, these data suggest that the TLR-4 pathway can play a key role in mediating physiological aortic homeostasis, by providing protection against pathogens and DAMPs, as well as in contributing to several pathological aorta phenotypes, including hypertension, MD, VR and atherosclerosis. These latter appear to have an onset in specific aortic regions. Today, the concept of a *regional heterogeneity of aorta diseases*, such as thoracic and abdominal aneurysms, is emerging [4,35, and 36].

4.3. The role of TLR-4 in the maintenance of aorta homeostasis

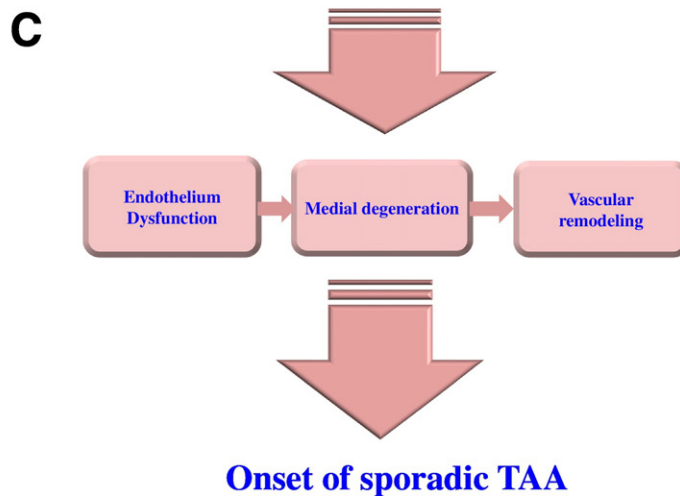
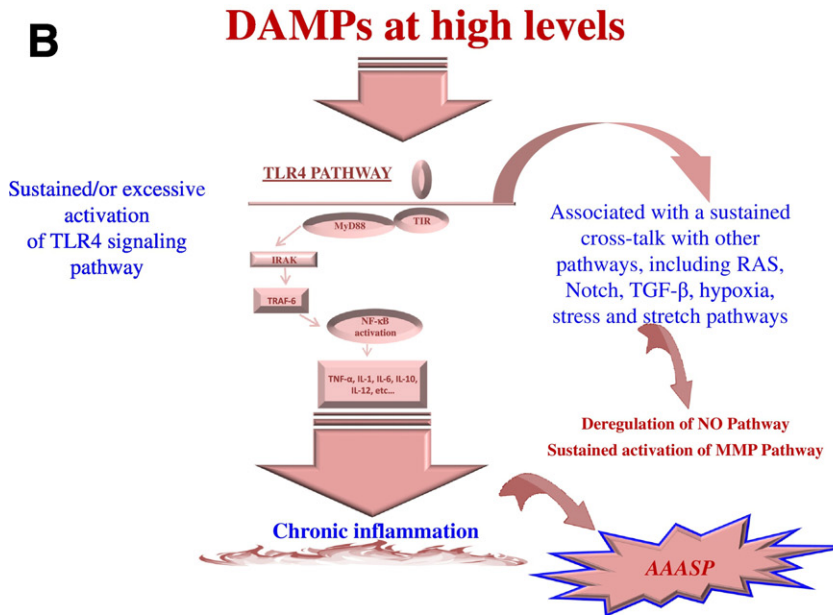
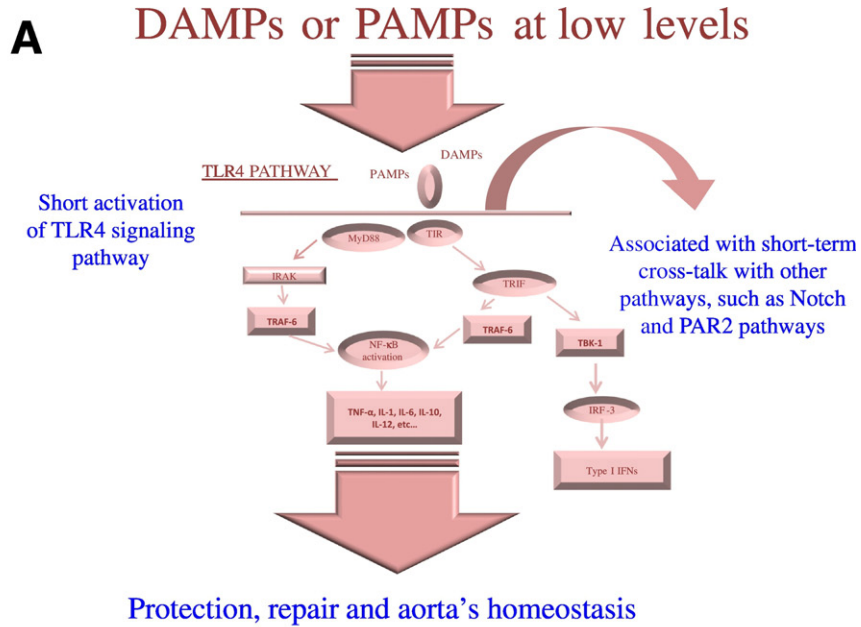
The role of TLR-4 in aortic health (see Fig. 5) and the disease has recently emerged. A recent survey reported the involvement of the TLR-4-mediated signaling pathway in the maintaining the integrity and normal turnover of endothelium. Specifically, He and colleagues analyzed human umbilical vein-derived endothelial progenitor cells (EPCs) and demonstrated that TLR-4 is expressed in these cells and it is involved in inducing proliferation and maintaining EPC stemness, probably through a cross-talk with other evolutionally conserved pathways, such as Notch pathway [88,89]. This interplay could allow a normal re-endothelialization and neovascularization, an increase in EPCs levels

in peripheral circulation, improving prognosis of aorta/cardiovascular diseases. Consequently, Li and colleagues [90], by investigating the EPC function in GroEL1 (*a heat shock protein 60 of Chlamydia pneumonia*)-administered EPCs in hind limb-ischemia in C57BL/6 and C57BL/10ScNj (a toll-like receptor 4 (TLR-4) mutation) mice and humans, provided with relevant evidence. Precisely, they observed that GroEL1 impaired the capillary density recovery, probably by TLR-4 in mice, including EPC mobilization and vessel formation as well as endothelial NO synthase (eNOS) expression in ischemic tissue. Additionally, the GroEL1 administration also impaired the migration and vasculogenesis of late EPCs in vitro and induced EPC senescence by activation of caspases, p38 MAPK and ERK1/2. Furthermore, it also decreased the expression of integrin $\alpha 1$, $-\alpha 2$, $-\beta 1$, $-\beta 3$ and E-selectin, but it induced inflammatory responses in EPCs. These findings propose that TLR-4 and impaired NO-related mechanisms could contribute to the reduced number and functional activity of EPCs in the presence of *C. pneumonia* GroEL1 and favor atherosclerosis onset [90]. Therefore, they suggest the relevance of this pathway in the protection against infective agents and in the maintenance of a favorable endothelial micro-environment for the physiological aortic function.

Another recent study performed by Bucci and colleagues in 2013 [91] reported that TLR-4 contributes to vascular homeostasis through a cross-talk with another signaling pathway, the Proteinase-activated receptor-2 (PAR-2). Specifically, they used thoracic aortas from both naïve and endotoxaemic rats for in vitro studies, and from TLR-4 (–/–) mice. PAR-2 expression, but not TLR-4, was increased in the aortas from endotoxaemic rats. PAR-2 AP-induced vascular relaxation was increased in aortic rings of LPS-treated rats. TLR-4 inhibitors, curcumin and resveratrol, reduced PAR-2 AP-induced vascular relaxation and PAR-2 AP-induced hypotension in both naïve and endotoxaemic rats. Moreover, in TLR-4 (–/–) aortic rings, expression of PAR-2 was reduced, and vasodilatation-induced by PAR-2 AP was impaired, with respect to wild-type mice, and yet after treatment with resveratrol and curcumin. Thus, they concluded that a cross-talk between PAR-2 and TLR-4 contributes to vascular homeostasis, creating a network with other stress and stretch pathways, including ACE, eNOS, and MMP pathways, [91] as demonstrated also in our model [8].

Overall, these data suggest that aorta homeostasis is the result of a very complex signaling network (see Fig. 5) between the TLR-4 signaling receptor itself pathway and other inflammatory and non-inflammatory pathways, some of which have been mentioned above, such as Notch pathway [89]. The growing evidence is also including other pathways, such as transforming growth factor- β (TGF- β) [92] and hypoxia pathways [93]. Among them, the Notch pathway (constituted by Notch receptors 1, 2, 3, and 4 and Delta-like ligands 1, 4 and Jagged 1, 2 ligands), an evolutionarily conserved pathway, has attracted the attention of researchers in the cardiovascular field [89]. The Notch pathway is fundamental to the development of all tissues, organs and systems of human body, as well as cardiovascular system, including aorta [94–97]. In aorta, Notch pathway is involved in the development of various aortic sections, and it appear to mediate the maturation of precursor cells from neural crests, and especially the differentiation of VSCMs [89,97,98]. However, in post-natal vasculature, Notch receptors 1, 2, 3, and 4 and Delta-like ligands 1, 4 and Jagged 1, 2 ligands are expressed in both ECs and VSMCs [89,97,98]. Precisely, Notch1 and Notch4 are predominant in aortic ECs and Notch3 in aortic VSMCs [89,98]. However, their complex and multiple roles, ranging from angiogenesis regulation, aorta homeostasis, to pathological conditions including inflammation, atherosclerosis, aorta diseases associated with the onset of both MD and VR, are still unclear. However, a close cross-talk with TLR-4 pathway has been proposed, which seems to justify its multitude of actions, and co-temporally contributes to the various

Fig. 5. A–C. TLR4 signaling pathway in homeostasis and sporadic TAA. In A, it describes a homeostasis condition determined by a short activation of this pathway, followed by a cross-talk with other pathways (see text). In B, it describes how high levels of DAMPs determine a sustained excessive activation of TLR4 signaling pathways, followed by a cross-talk with TGF- β , hypoxia, Notch, NO, MMP, NF- κ B pathways. As a result, endothelium dysfunction, hypertension, medial degeneration, vascular remodeling and sporadic TAA are induced (see panel C).



biological effects of TLR-4 in the cardiovascular system, as well as in the aorta [89]. Current evidence supports this. For example, Zeng and colleagues showed that Notch-1 mediates the pro-osteogenic response to TLR-4 stimulation in human aortic valve interstitial cells (AVICs) [94]. Thus, they concluded that this pathway could be a potential therapeutic target for prevention of progression of calcific aortic valve disease (CAVD) [94]. In another study performed in 2014 [95], the same group tested the hypothesis that oxidized low-density lipoprotein (oxLDL) increases the osteogenic responses in human AVICs, by modulating the TLR-4-NF- κ B pathway and Notch1 activation. AVICs were isolated from normal human aortic valves and treated with LPS (0.1 μ g/ml), oxLDL (20 μ g/ml) or LPS plus oxLDL for 48 h. The results obtained demonstrated that oxLDL up-regulated the bone morphogenetic protein-2 expression in human AVICs and synergized with LPS to induce high AVIC osteogenic responses. Hence, the authors have shown that oxLDL exert their effect through modulation of the Notch1-TLR-4-NF- κ B signaling cascade [95]. Moreover, Kerr and colleagues demonstrated in mice that the stability and function of adult vasculature is supported by Akt (a kinase protein representing a TLR-4-signaling adaptor [99])/Jagged1 signaling axis in endothelium [100].

This cross-talk between TLR-4 and Notch pathways (highlighted in other age-related diseases, as is largely underlined in our recent review of the Notch pathway [89], and with other above mentioned pathways) supports both the versatility of action and function of TLR-4 itself pathway to induce several cellular responses, and pleiotropic effects in the aorta (and specifically in the various aortic sections). These last reflect not only the complexity and the fundamental features of this pathway (as stressed above), but also its ability to be modulated and regulated by genetic variants (as reported above), but also by intrinsic downstream pathways and extrinsic molecules. To date, several regulators have been identified. They are involved in inhibiting an over- or unnecessary activation of TLR-4 pathway, as well as other PRRs. In Table 4, many of these molecules are listed and described [101–104].

In addition, the recent research points that miRNAs (i.e. miR21, miR-let7, miR146a, miR-155, and miR-126) could play important roles in modulating gene expression of TLR-4 pathway in the aortic wall and proteinic levels. miRNAs seem to have dual roles: a) the activation of TLR-4-mediated pathway activation and b) the inhibition of NF- κ B signaling, in a complex scenario where low and chronic inflammation prevails, and probably also sustained by cell senescence secretome. miRNAs inhibition effect probably belongs to the various levels of anti-inflammatory pathways that have evolved to lower the TLR-4 signaling pathway to prevent cell and aortic destruction [105–107].

In the complex, all these data reveal an emerging aspect of TLR-4 signaling pathway that is its involvement in aortic physiology. This may suggest new prospects for the use of TLR-4-related mechanisms to modulate the EPCs production for clinical use [34]. Co-temporally, they point that TLR-4 signaling pathway has a multi-faceted biology and complex function, able to mediate not only protective biological effects for aorta health, but also dangerous biological functions, including the onset of aortic diseases (for this reason we have defined the TLR4 pathway as *the signaling pathway from the double-face*, see above) [8].

5. Role of TLR-4 pathway in aneurysms (sporadic TAA)?

A growing number of studies report promising data on the role of TLR-4 signaling pathway in human aortic diseases, such as aneurysms (see Fig. 5B and C). The first evidence derives from data on the cellular expression and involvement of the TLR-4 signaling pathway in atherosclerotic arteries, and precisely on ECs, macrophages [108,109] and adventitial fibroblasts of these arteries [110]. In 2004, the group of De Kleijn [111], using a femoral artery cuff model in the atherosclerotic ApoE3 (Leiden) transgenic mouse and inducing plaque formation through LPS-mediated TLR-4 activation, demonstrated that the TLR-4 signaling pathway is involved in outward arterial remodeling, probably through up-regulation of TLR-4 itself and its ligands. In 2006, Lin and colleagues [112], by using

cell cultures of human aortic smooth muscle cells (HASMCs) from vessels obtained from Zeal and white rabbits treated or without intravenous injections of LPS (110 ng/kg), evidenced that the stimulation of HASMCs with LPS significantly increased the TLR-4 expression. Increased TLR-4 expression was observed to be controlled by NADPH oxidase activation, mRNA stabilization, and MAPK signaling pathways. As a result, this promoted a HASMCs inflammatory phenotype and vascular remodeling [112]. In addition, in 2007, Schultz and colleagues [113], using human VSMCs cell cultures derived from digestion of human coronary artery or pulmonary artery, detected that autocrine actions of endogenous interleukin (IL)-1 α , mediated at least in part via IL-1RI signaling, contributed to a pro-proliferative and proinflammatory phenotypic shift in TLR-4-activated human VSMCs. As a consequence, they suggested a pathogenic role of TLR-4 pathway in vascular disorders. Based on this evidence, Pi and colleagues [114] recently investigated the essential role of reactive oxygen species (ROS) in inducing vascular TLR-4 expression and neointimal formation. Precisely, they tested the hypothesis that ROS promote VSMC proliferation and migration by enhancing TLR-4-mediated inflammation, and ultimately leading to intimal hyperplasia after injury. Reciprocally, inhibition of ROS generation might restrain this progress. As a result, male TLR-4-deficient mice (C57BL/6 J background) and wild-type mice (WT, C57BL/6 J) at 8–10 weeks of age were used to obtain VSMCs from thoracic aorta. The generation of ROS was pharmacologically manipulated respectively by Platelet-derived growth factor (PDGF)-BB and apocynin, respectively. They observed that VSMCs from thoracic aorta exposed to PDGF-BB showed significant increase in the generation of ROS, which was effectively suppressed by apocynin. Inhibition of ROS attenuated both TLR-4 and the expression of proinflammatory cytokines in VSMCs, and was accompanied by a significant inhibition of VSMC proliferation and migration, and neointimal formation. Thus, they provided evidence that ROS may be a key trigger in evocating both TLR-4-mediated vascular inflammation and intimal hyperplasia. Contemporally, they suggested that the inhibition of the primary source of ROS could mitigate the TLR-4-mediated proinflammatory and proliferative phenotype of VSMCs, and reduce the detrimental arterial remodeling and inflammatory reaction (i.e. that observed in the early stages of atherosclerosis) [114]. In addition, in 2012 the group of Garcia-Rodriguez [115] investigated TLR-mediated inflammation and osteogenesis in interstitial human cells, isolated from stenotic and non-stenotic aortic valves and stimulated with viral and Gram-negative bacteria molecular patterns. TLR- expression and signaling were evaluated by several assays, including quantitative-PCR and flow cytometry, to Western blot analysis, ELISA, and cytokine arrays. Osteogenesis was detected by determining alkaline phosphatase activity. The results obtained demonstrated that interstitial cells from control valves expressed high levels of TLR mRNA, and precisely they showed higher levels of TLR-4 pathway mRNA. In contrast, the cells from stenotic valves had higher TLR-4 and TLR2 and lower TLR5 and TLR9 transcript levels. Furthermore, analysing the pro-inflammatory pathways, they also detected that TLR-4, TLR2 and TLR3 ligands induced an early activation of NF- κ B and p38 MAPK in the cells from control and stenotic valves. Remarkably, when TLR sensing viral patterns were studied, they also assessed a sustained TLR3-mediated activation of NF- κ B, a κ B independent induction of catalytically active cyclooxygenase (COX)-2 and ICAM expression, and induction of a lot of chemokines. Moreover, they showed that TLR3 and TLR-4 agonists induced alkaline phosphatase expression and activity. Thus, they concluded that exposure of aortic valve interstitial cells to viral and Gram-negative bacteria molecular patterns induces distinct and long-term TLR-mediated pro-inflammatory and pro-osteogenic responses, likely relevant in the pathogenesis of degenerative aortic stenosis [115].

These encouraging data have led several research groups to examine the role of TLR-4 signaling pathway in aorta aging, and in vascular pathological conditions that is VR and MD, significantly associated with the

onset of sporadic TAA [4]. In 2012, Song and colleagues [116] analyzed the inflammatory response of aortas from young (2–4 months) and aged (16–18 months) mice under no stimulatory conditions. They

observed increased IL-6 basal levels in aged aortas. In addition, aged aortic VSMCs showed a higher basal release of IL-6 than young VSMCs. Using gene and protein expression analysis, they also showed that aged VSMCs

Table 4
Intrinsic and extrinsic or exogenous regulators of TLR-4 pathway [101–104].

Regulators	Type	Action
sMyD88	Intrinsic molecule	The short form of MyD88 (sMyD88) substitutes MyD88 but cannot send signals downstream
Toll-interacting protein (Tollip)	Intrinsic molecule able to interact with several members of the TIR superfamily, including TLR2 and TLR4	Tollip interacts with IRAK to decrease phosphorylation
A20	Intrinsic molecule, initially identified as a TNF-induced zinc-finger protein A20 expression is rapidly induced by both TLR4 ligands, LPS and TNF, and is expressed in many cell types, which suggests that it is involved in regulating TLR function	A20 deubiquitylates TRAF6
Silenced suppressor of cytokine signaling 1 (SOCS1)	Intrinsic molecule involved in negative regulation of cytokines that signal through the JAK/STAT3 pathway	SOCS1 regulates phosphorylation of I κ B α , p38, and JNK
TRIF-related adaptor molecule (TRAM)	Intrinsic molecule involved in multiple signaling cascades, including TLR-4 signaling cascade	TRAM adaptor with GOLD domain (TAG), identified as a variant of TRAM, competes with TRAM for TRIF-domain-containing adaptor protein-inducing IFN- β (TRIF) binding and inhibits the dependent pathway. TAG localizes to the late endosomes and is required for TLR4 degradation after lipopolysaccharide (LPS) treatment, indicating that TAG may mediate destabilization of TLR4 by delivery to lysosomes, as well as inhibiting TRIF binding
Sterile alpha- and armadillo-motif-containing protein (SARM)	Intrinsic molecule: another TIR domain containing protein	SARM can block TRIF complex formation by directly binding to TRIF after LPS treatment
Interferon (IFN) regulatory factor (IRF) 4	Intrinsic molecule involved in the induction of a set of TLR-inducible genes	IRF5 directly interacts with MyD88 to induce a set of TLR-inducible genes. IRF4 is induced by TLR activation and competes with IRF5 for binding to MyD88, resulting in shutdown of IRF5-dependent gene induction. IRF4-deficient mice are hypersensitive to DNA-induced shock accompanied by increased cytokine production
A tumour necrosis factor, alpha-induced protein 8 (TNFAIP8), TNFAIP8-like 2	Intrinsic molecule associated with multiple signaling cascades, including TLR-4 signaling cascade	It binds to caspase 8 and regulates activator protein (AP)-1 and NF- κ B activation
Nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family member \times 1 (NLRX1)	Intrinsic molecule involved in inflammasome activation	NLRX1 undergoes K63-linked polyubiquitination after LPS treatment and dissociates from TRAF6, resulting in binding to the activated kinase domain of IKK β via the leucine-rich repeat(LRR) domain
Post-translational modifications such as phosphorylation and ubiquitination	Intrinsic mechanisms	Post-translational modifications such as phosphorylation and ubiquitination also play important roles in signal transduction by regulating interactions among adaptor proteins.
The orphan nuclear receptor, small heterodimer partner (SHP, also known as NROB2)	Intrinsic molecule	SHP (also known as NROB2) has been identified as a negative regulator of TLR signaling by inhibiting TRAF6 ubiquitination. Precisely, it has also been demonstrated that TLR-4 stimulation induces SHP expression through AMP-activated protein kinase (AMPK) activation-dependent intracellular Ca $^{2+}$ influx mediated by TLR-4
Mitogen and stress activated protein kinase (MSK) 1 and 2 activated in the mitogen-activated protein kinase (MAPK) cascade	Intrinsic molecules involved in multiple signaling cascades, including TLR-4 signaling cascade	MSK1 and MSK2 limit the proinflammatory effects of TLR4 signaling MSK1 and MSK2 induce the binding of phosphorylated transcription factors cAMP responsive element binding protein 1 (CREB) and activating transcription factor 1 (ATF1) to the promoters of the anti-inflammatory cytokine IL-10
TGF- β -activated kinase 1 (TAK1)	Intrinsic molecules involved in multiple signaling cascades, including TLR-4 signaling cascade	TAK1 negatively controls p38 activation in myeloid cells (neutrophils and macrophages)
Several phosphatases and deubiquitination enzymes (DUBs)	Intrinsic molecules involved in TLR-4 signaling cascade	The Src homology 2 domain-containing protein tyrosine phosphatase-1 and -2 (SHP-1, -2) are involved in TLR signaling. SHP-1 suppresses IRAK1 and IRAK2 activities, resulting in decreased production of proinflammatory cytokines and increased production of type I IFN. SHP-2 negatively regulates TRIF-dependent type I IFN production
DUBA (deubiquitinating enzyme A)	Intrinsic molecules involved in TLR-4 signaling cascade	DUBA has been identified as a negative regulator of type I IFN production
Viral and bacterial Proteins (including Hepatitis C virus (HCV) protein NS3-4A; Vaccinia virus (VACV) protein A46R; IpaH9.8 protein from Shigella flexneri; Mycobacterium tuberculosis wall component)	Exogenous molecules	They induce inhibition of TLR-4 signaling cascade
Soluble TLR4 molecules	Extrinsic molecules	They act as decoy receptors inhibiting TLR-4 signaling pathway
TAM receptors	Extrinsic molecules	TAM receptors are involved in the inhibition evoking the expression of regulator downstream molecules of TLR-4 cascades

also had up-regulation of chemokines (e.g., CCL2), adhesion molecules (e.g., intracellular adhesion molecule 1), and mainly of TLR-4 pathway. The group of Eissler and colleagues [117] also evidenced that age-related hypertension is able to induce vascular inflammation via TLR-4 expression. This evidence has recently been confirmed by other groups. In 2014, McCarthy and colleagues [118], established that DAMPs, released by damaged tissues and entered in circulation, activate TLRs, and in particular the TLR-4 pathway, in vascular somatic cells of the, and consequently induce inflammation, vasoreactivity, and vascular remodeling which result in hypertension. This was understood by Sollinger and colleagues [119], using TLR-4 (–/–) and wild-type mice. De Batista and colleagues [120] reported a key role of AngII in contributing to TLR-4 up-regulation, VR and hypertension in aortas from Wistar rats treated with a non-specific IgG (1 µg/day) and adults male rats with spontaneous hypertensive (SHRs) treated with losartan (15 mg/kg·day), non-specific IgG or neutralizing antibody anti-TLR-4 (1 µg/day). In particular, they have shown that SHR treatment with the anti-TLR-4 antibody reduced blood pressure, heart rate and phenylephrine-induced contraction, while improved the impaired acetylcholine-induced relaxation. Furthermore, it increased the phenylephrine contraction after endothelium removal and abolished the inhibitory effects of apocynin and catalase on the phenylephrine-induced response, as well as its enhancing effect of acetylcholine-induced relaxation. In SHR VSMCs, AngII increased TLR-4 mRNA levels, while losartan reverted them. Moreover, the use of CLI-095, a TLR-4 inhibitor, mitigated the increases in NAD (P) H oxidase activity, superoxide anion production, migration and proliferation, induced by AngII. Thus, the Batista's group concluded that the TLR-4 pathway activation due to increased RAS activity is involved in hypertension, and this pathway contributes to the endothelial dysfunction associated with this pathology, by inducing oxidative stress [120]. These findings were validated in 2015 by Hernandez's group [121]. Precisely, they examined whether TLR-4 activation likely contributed to Ang-II-induced-hypertension and the related vascular structural, mechanical and functional alterations. For this purpose, AngII was infused (1.44 mg kg⁻¹ day⁻¹, s.c.) for 2 weeks in C57BL6 mice, treated with a neutralizing anti-TLR-4 antibody or IgG (1 µg day⁻¹). The values of systolic blood pressure (SBP) and the levels of aortic cytokines were measured. The structural, mechanical and contractile properties of aortic and mesenteric arterial segments have been measured by myography and histology. RT-PCR and Western blotting were also used to evaluate tissues and cultured VSMCs from hypertensive rats (SHR). The findings obtained demonstrated that aortic TLR-4 mRNA levels were elevated after AngII infusion. In contrast, the administration of anti-TLR-4 antibody to AngII-treated mice reported them to physiological values, but it increased SBP and TNF-α, IL-6 and CCL2 levels. In addition, it resulted in vascular and structural changes, with altered aortic phenylephrine and Ach induced responses and increased NOX-1 mRNA levels, superoxide anion production and NAD(P)H oxidase activity and the effects of catalase, apocynin on vascular responses. Furthermore, it reduced the NO release and effects of L-NAME on phenylephrine-induced contraction. In VSMCs, the MyD88 inhibitor ST-2825 reduced NAD (P) H oxidase activity induced by AngII. The TLR-4 inhibitor CLI-095 reduced the increase of expression of phospho-JNK1/2 and p65 NF-κB subunit nuclear protein induced by AngII. These relevant results have suggested that TLR-4 up-regulation associated with both hypertension and evocation of mechanisms involving oxidative stress is likely able to contribute to several pathological conditions, including inflammation, endothelial dysfunction, vascular remodeling and stiffness. For validating these data, in 2015 Bomfim and colleagues [122] treated SHR and Wistar rats with anti-TLR-4 antibody (1 µg/day) or unspecific IgG for 15 days (i.p.). Anti-TLR-4 treatment resulted in the production of ROS and IL-6 expression in mesenteric resistance arteries from SHR, when compared with IgG-treated SHR. Furthermore, the anti-TLR-4 treatment also abolished the increased vascular responsiveness to noradrenalin in IgG-treated SHR, and the inhibition of NFκB decreased noradrenalin responses only in IgG-treated SHR. Mesenteric arteries from SHR treated with anti-

TLR-4 showed decreased expression of MyD88, but not TRIF, key molecules in TLR-4 signaling. Phosphorylation of p38 and NF-κB p65 was reduced in arteries from anti-TLR-4-treated SHR versus IgG-treated SHR. Hence, these results suggested that TLR-4/NFκB signaling pathway is a key role in hypertension and vascular inflammatory process [122].

Other investigations have been conducted by other research groups for identifying the molecular mechanisms mediated by TLR-4 pathway in inducing aortic VR and MD, which are pathological entities significantly associated with sporadic TAA, as above mentioned (see Fig. 5 B and C). The group of Gozales-Ramos [123] pointed out that circulating Hsp70 associated with increased aorta damage, regulates the profibrotic response of human aorta SMCs through increased TGF-1 expression, evoked by TLR-4-mediated signaling pathway. In addition, Li and colleagues [124] reported the key role of TLR-4-mediated signaling pathway in regulating the MMP-9 expression in HASMCs. Furthermore, a recent study has demonstrated in apolipoprotein E-deficient mice that it is possible to limit the inflammatory process, blocking TLR-4/c-Jun N terminal kinase signaling pathway with Rosiglitazone (a family of drugs acting as agonists of the nuclear peroxisome proliferator-activated receptors-PPARs) in the initiation stages of aortic aneurysm development [125].

Based on these relevant findings, we first assessed the role of ten genetic variants (related to genes codifying molecules) of the TLR-4-mediated signaling pathway in the susceptibility for sporadic TAA and dissection [4,8,126]. Interestingly, we found that their combined genotype was significantly represented in sporadic TAA than controls. In subsequent investigations, we also have shown that cases with this risk genotype showed a higher systemic inflammatory mediator levels [8, 126], a significant inflammatory/immune infiltrate [8,126], a typical MD phenotype [127], a lower telomere length [128,129] and positive correlations with histopathological abnormalities [126,127], hypertension, smoking, and aging [8,126–129]. Consequently, our findings (even if prevalently of associated type) led us to suggest the key role of TLR-4 pathway in the onset of this disease [4,8]. Moreover, they support actual growing evidence (as reported above), the suggestion and consideration of the studies above described. Accordingly, it proposes (see Fig. 5B and C) that the activation of TLR-4-mediated signaling pathway, expressed both on EC and VSMC cells, could determine the activation or deregulation of ACE, NO, MMP, TGF-β pathways (associated with endothelium dysfunction, extracellular matrix remodeling and chronic inflammation) and as a consequence to induce the onset of VR and MD, and sporadic TAA, as their complication. As a result, we postulated (as above reported) a sporadic TAA model, which we defined as *model of the signaling pathway from the double-face*, given its features (see Fig. 4 of Ruvolo et al., 2014 study [8]; <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4120489/>). We hope it could lead several researchers to perform additional investigations useful to clarify the complexity of this pathology. Certainly, future studies and additional efforts are necessary, as well as a more complex combination of investigations based on genetic, transcriptomic, proteomic, metabolomic, microbiomic and epigenetic evaluations, since each individual is the result of the sophisticated interplay between environmental factors and its genome, transcriptome, proteome, metabolome, microbiome, epigenome, exposome (see Fig. 6). This approach might be very innovative, and it could provide valuable insights about sporadic TAA pathophysiology, although it is very difficult to obtain human tissue aorta samples and appropriate controls. As an alternative solution (and more feasible), research cardiovascular community proposes the use of appropriate animal models to study human aorta diseases, i.e. sporadic TAA, providing the means to test new pharmacological interventions. However, there is a considerable debate in the field of aneurysm's research on disadvantages and advantages associated with the use of rat and mouse models, in which aneurysms can be generated in several experimental settings [130,131]. Animal models have provided useful information. However, a model, which replicates the chronic disease in humans, remains to be produced. Recently, large mammal models have been used in the study of aortic aneurysms, including dog, pig,

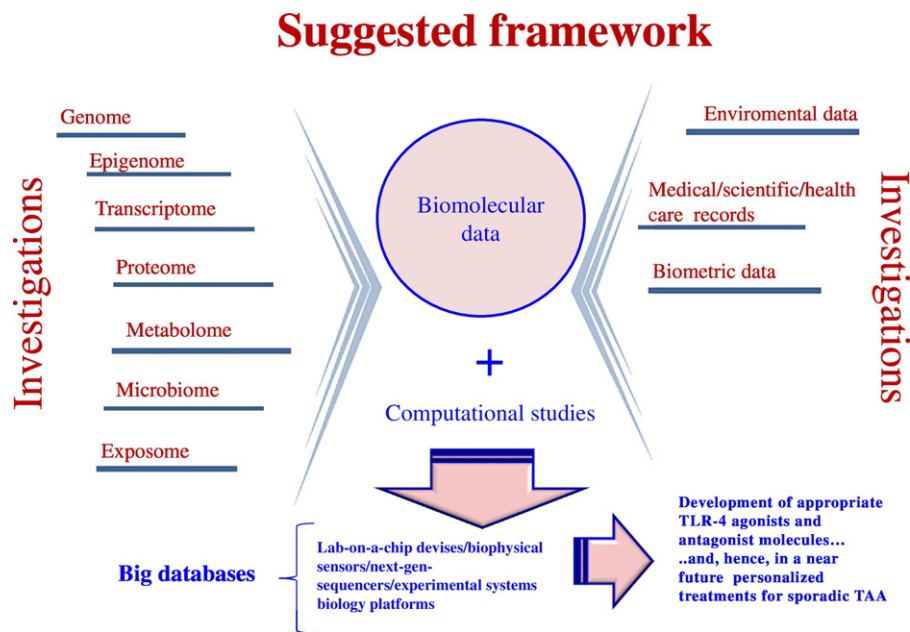


Fig. 6. A suggested framework: integration of genome, transcriptome, proteome, metabolome, microbiome, epigenome, exposome evaluations with computational investigations. Since each individual is the result of the sophisticated interplay between environmental factors and its genome, transcriptome, proteome, metabolome, microbiome, epigenome, exposome, we here suggest that it is necessary to perform a more complex combination of investigations based on genetic, transcriptomic, proteomic, metabolomic, microbiomic and epigenomic evaluations, for obtaining interesting data in the study of sporadic TAA. In addition, computational investigations are also recommended, as well as collecting environmental and biometric data, medical/scientific/health care records. Thus, we propose that the integration of all data, obtained in this large panel of assays, and their elaboration might lead to the development of appropriate agonists, antagonists, inhibitors of TLR-4 signaling pathway, which might be used in a near future as personalized treatments for sporadic TAA (until now inexistent), facilitating the management and outcome of this insidious deadly pathology.

rabbit, sheep and turkey [130,131]. But they show other problems for which the solutions are far less evident. Firstly, large animal models are very expensive, and sometimes prohibitive. In addition, they also need a very intensive work environment, requiring a large and multidisciplinary greater care. As a result, mouse models appear to be the best animal model in aneurysm study so far, as they are relatively small, easy to handle, and not expensive. In addition, they can be genetically manipulated to mimic human diseases, by generating “knockout” or “knock-in” models and increasing their longevity in a rapidly evolving field [130,131]. Thus, this might facilitate the identification of potential mechanisms and pathways related to aneurysms, such as sporadic TAA. Accordingly, they could consent to investigate the relevance of TLR-4 signaling pathway in aorta, and precisely in both aorta health and diseases, sporadic TAA included (as reported above). Thus, there is a great potential for animal studies to improve our understanding of sporadic TAA pathophysiology, with the aim of improving current patient management strategies.

6. Therapeutic role of TLR-4 pathway: from experimental data to development of innovative therapies

Overall, the data, above stressed, highlighted the relevant role of the TLR-4 signaling pathway in aortic diseases, i.e. sporadic TAA, although unclear responses and a number of gaps have to be resolved. However, they encourage the approach of *translational medicine* (TM), an emerging and multidisciplinary area and a new tool for improving human health by reducing the incidence, morbidity and mortality of the disease [132–135]. Translational research turns scientific discoveries found in the laboratory in ways to prevent diagnosis or treat diseases (i.e. *personalized therapies*). In the specific case of aneurysms, translational scientists are primarily focusing on their interest on infilling knowledge of related TAA phenotypes (i.e. hypertension, VR, MD and its complications, such as sporadic TAA), obtained from in vitro and experimental animal models, with pathways of disease identified in humans, such as the TLR-4 pathway. The identification of susceptibility genes, associated with these pathways of disease and which allows patients to achieve personalized treatments, is their subsequent purposes.

In regards to TLR-4 pathway, biotechnology companies are developing various drugs (i.e. agonists or antagonists), ranging from proteins to metal ions, such as pharmacological treatments to modulate its activation under atherosclerosis, ischemic heart disease, heart failure, ischemic reperfusion injury, and aneurysm [136,137]. A considerable number of them are listed in Table 5, together with their effects [136,137]. With regard to the latter, some have been reported in the studies described [115, 120–125] in the previous paragraph. Their action has shown that both the TLR-4 activation and inhibition may have beneficial effects in various conditions, reducing tissue immune inflammatory responses [115, 120–125, 136, and 137]. This result can be explained with the diverse interaction of the TLR-4 pathway with different pathways, expressed differently in various types of experimental models used, as mentioned above. However, the TLR-4 modulators and their biological effects seem to be dependent on other factors, including firstly the timing of administration during the various stages of disease onset and progression, as well as the age and genetic background of animal models used at the time of experimentation. In this regards, we have highlighted in a recent review [11], that there are age-related defects in TLR-4 function and expression, as reported in human studies, although these studies have did not led conclusive data, being limited by the heterogeneity of epidemiological and laboratory methods. This could also be valid for the animals used as a model of study. Furthermore, as mentioned above, the TLR-4 function and expression are modulated not only by genetic variants and haplotypes [11,85,86] but also by environmental factors (such as diet [138–141], mite allergens [142], air pollution [143]), and their cross-interaction with microbiota [141], which may remain in a healthy state or show alterations (i.e. dysbiosis and consequent endotoxemia associated with age or obesity) [144,145] and consequent epigenetic changes [146,147]. Thus, many limitations and concerns emerge. In addition, another relevant limitation derives from the type of experimentation. Precisely, agonists and antagonists have been mainly analyzed in cell cultures and animal models [136,137]. Thus, only preclinical studies exist until now, and no human trials have been performed. As a consequence, the encouraging data obtained may not be confirmed in humans. In fact, there are many concerns in this field. The first concern comes

Table 5
Pharmacological Agonists and Antagonists and natural small molecules modulating TLR-4 signaling pathway [115,120–125,136,137].

Molecules	Action	Diseases	Preclinical studies
BCG	TLR-4 agonist	Cardiac hypertrophy	Mouse
Eritoran	TLR-4 antagonist	Cardiac ischemia; Cardiac Hypertrophy Renal injury	Rat; Mouse
Valsartan	TLR-4 inhibitor	Cardiac ischemia	Rat
Atorvastatin	TLR-4 inhibitor	Cardiac ischemia	In vitro
Losartan	TLR-4 inhibitor	Cardiac Failure	Mouse
Fluvastatin	TLR-4 inhibitor	Cardiac Failure	In vitro
Cinnamic acid	TLR-4 inhibitor	Myocarditis	Mouse
Geldanamycin	TLR-4 inhibitor	Myocardial inflammation	Mouse
Baicalin	TLR-4 inhibitor	Brain ischemia	Rat
Oxymatrine	TLR-4 inhibitor	Brain ischemia	Rat
Progesterone	TLR-4 inhibitor	Brain ischemia	Rat
Gynkgobiloba	TLR-4 inhibitor	Brain ischemia	Rat
Picoside 2	TLR4 inhibitor	Brain ischemia	Rat
Oestrogen	TLR4 inhibitor	Lung/ischemia reperfusion	Mouse
Dexmedetomidine	TLR4 inhibitor	Renal ischemia	Mouse
Melatonin	TLR4 inhibitor	Liver/ischemia reperfusion	Rat
Surforaphane	TLR4 inhibitors, by blocking oligomerization of the receptor	Inflammatory diseases	Rat; Mouse, in vitro
Cinnamaldehyde			
Curcumin			
Epigallocatechin gallate (EGCG), resveratrol and certain flavonoids such as luteolin, quercetin, chrysin, and eriodictyol	TLR4 inhibitors: have the ability to decrease kinase activity of TBK1 activating IRF	Inflammatory diseases	Rat; Mouse, in vitro
Xanthohumol (XN)	TLR4 inhibitor: XN is anti-inflammatory natural product from hops and beer, it can block the TLR4 signaling by binding to MD-2 directly	Inflammatory diseases	Rat; Mouse, in vitro
Ligustrazine, a natural alkaloid compound	TLR4 inhibitor	Inflammatory diseases	Rat; Mouse, in vitro

from the action of agonist and antagonist molecules, which is debated. A large number of researchers claim that only a subset of them could probably activate or inhibit directly the TLR-4 pathway. Their structural diversity is enormous, and this suggests promiscuity in their TLR-4 binding capacity, without specificity of action, and rather a pleiotropic effect on different receptors. In this regard, Mancek-Keber and Jerala [148] have proposed three postulates to distinguish direct agonists from indirect activators: (1) the agonist requires the TLR-4/MD-2 receptor complex; (2) either synthetically or in situ, they must activate the receptor complex in order to eliminate artefacts from other agonists; and (3) a specific molecular interaction between the agonist and TLR-4/MD-2 must be identified. Furthermore, in 2016 the group of Martin-Santamaria [149, 150] proposed to use computational approaches [149] and big databases [150] for the identification of all atomic details in the TLR-4 receptor structure itself and the ligand-receptor interactions of different modulators to develop novel agonists and antagonists with promising biomedical applications, to reduce toxic effects, and improve drug-like properties, fine-tuning of relative potency of agonists and antagonists, binding capacity and specificity.

Similar considerations may also be valid for small natural molecules, such as curcumin, cinnamaldehyde and sulforaphane, which have been reported to mediate the anti-TLR4 dimerization effects, as well as for resveratrol and some flavonoids (such as EGCG, luteolin, quercetin, chrysin, and eriodictyol), that have been reported to inhibit TBK1 kinase activity (a downstream TLR4 signaling kinase), resulting in a decreased IRF3 activation, a TLR4 signaling adaptator, and target gene expression [101–104,150] (see Table 5).

On the one hand, these observations support the idea that these molecules can find clinical implementations in the near future to

facilitate management and to develop treatments so far inexistent for aortic aneurysms, such as sporadic TAA. On the other hand, they suggest that the solution can be in putting together the investigations, suggested in the preceding paragraph, with the evaluations of TLR-4 modulators described above to facilitate the development of personalized treatments for aortic diseases, i.e. sporadic TAA (see Fig. 6). Probably, looking with new eyes the investigations and the results obtained might be of help in order to make new discoveries, although the way of research to practice is still long and difficult, as suggested in our studies [4,8].

7. Conclusions and future perspectives

The human aorta is the main vessel, which carries blood pumped from the left ventricle to the systemic circulation. Thus, aorta has a decisive role in contributing to the health of all tissues, organs and systems of the human body, providing oxygen and nutrients. A very sophisticated control regulates its activity and contributes to maintaining a fine homeostasis. This regulation (similarly to that regulating the entire cardiovascular system) is dominated by the central nervous system (CNS) [151], the knowledge of which in the past 20 years has significantly evolved. This control is also supported, or rather induced by autonomic nervous system (ANS) [152], which completes the primary ring of homeostasis body's control along with the immune and endocrine systems. These latter, through molecular pathways of innate signaling (likely TLR-4 signaling pathway) and hormones (i.e. reproductive hormones), which were not classically viewed as CV (aorta included) signaling pathways, mediate both ANS and CNS target effects in traditional and not traditional manner [152]. In fact, they also act via

membrane receptor-independent signaling mechanisms and ROS, all of which have been shown to have profound effects on the central control of blood pressure and activity of stress and stretch signaling pathways of aortic EC and VSMC cells [153,154]. In addition, recent investigations carried out in particular on mice have shown that the activation of mechanical and strain stress (i.e. ACE, TGF- β) induces inflammation and degeneration, and it increases hypertension, providing insight into formation and progression of aorta diseases, including sporadic TAA and dissection (see Figs. 2 and 5 B and C) [154,155]. In turn, chronic inflammation, such as chronic signal, vascular aging and CVD onset, such as sporadic TAA, has been shown (see Fig. 5 B and C) [34,36, and 37,156].

Overall, these observations suggest that inflammation is now recognized as the basic mechanism of aging of the cardiovascular system, including the related diseases, as sporadic TAAs [156,157]. On the other hand, a longitudinal study performed in 2016 in a very wide range of age from 45 to 115 years (1554 enrolled individuals), including unprecedented large numbers of extremely old, suggested that inflammation is an important aging driver and its reduced levels could be an optimal biomarker for a successful aging with respect to telomere length [158]. Thus, future pharmacological intervention on inflammation may be amenable [158]. Our data gathered for over 10 years in Sicilian centenarians are in agreement with these results [79]. Based on results obtained, we, indeed, suggest that longevity is the result of an optimal performance of the immune system and an over-expression of anti-inflammatory sequence variants of immune/inflammatory genes. We mutually demonstrate that age-related diseases are evoked by chronic inflammation and by an over-representation of pro-inflammatory gene variants of immune/inflammatory genes [79]. Furthermore, we underline that an intricate inflammatory network is involved in the complex pathophysiology of age-related diseases, where the TLR-4 signaling pathway acts as hub [9,11]. Likewise, here, we point the key role of the TLR-4 signaling pathway in aorta health and diseases, and particularly in sporadic TAA, highlighting an onset model [4,8]. In addition, we point out that its versatility of action and function and its consequent pleiotropic effects are the result of a complex cross-talk with other signaling pathways, such as the Notch pathway, as we have highlighted in a recent review [89]. Certainly, further investigations to validate and confirm the role of the TLR-4 signaling pathway in sporadic TAA, are needed. However, it should be noted that this report may lead several researchers to perform additional studies to clear the complex puzzle of this pathology. Future studies and additional efforts are imperative. Here, we propose that a combination of analyses (see Fig. 6) can be helpful. The results obtained could favor the development of personalized treatments for sporadic TAA, until now inexistent. In this context, investigations that clarify the effects of environmental triggers, such as diet, allergens, air pollution, in the pathophysiology of aorta aging and diseases (i.e. sporadic TAA) through activation of TLR-4 pathway, including the intriguing link between the intestinal microbiota and CVD mediated by the TLR4-pathway, might be helpful (see Fig. 6).

To achieve this goal, our message is “*We must not give up in the face of many difficulties, that we face in performing our research ... but above all we must not forget that the ultimate goal of our experiments should be the good for the man and not the love for the success ... motivated exclusively by this ... the perseverance, collaboration and honest activity, together with the necessary funding, will allow us to convert assumptions into certainties*”.

Competing interests

The authors declare that there are not conflicts of interests.

Author contribution

Dr. Balistreri firstly contributed to conception and design, and in drafting the paper. Dr. Madonna was firstly involved in contributing to drafting the paper. Drs. Balistreri and Madonna also contributed to the

critical revision. All authors participated in the study, and they read and approved the final version of the paper.

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