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SINGLE NUCLEOTIDE POLYMORPHISMS AND microRNAs AFFECTING PTX3 PRODUCTION

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Dottorando: Elisa BARBATI
Matricola: R08013

Tutore: Dr.ssa Cecilia GARLANDA
Coordinatore del dottorato: Prof. Alberto MANTOVANI

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Summary

Pentraxins are fluid phase pattern recognition receptors phylogenetically conserved from arachnids to mammals. Based on the primary structure, pentraxins are divided in short pentraxins, like the C-reactive protein and the serum amyloid P, and long pentraxins, like PTX3. PTX3 is expressed by several cell types, including mononuclear phagocytes, myeloid dendritic cells, fibroblasts, smooth muscle cells and endothelial cells in response to pro-inflammatory cytokines and microbial components. This molecule is involved in innate immunity, tissue remodelling, female fertility and in tuning the inflammatory response. In particular, PTX3 plays a protective role in cardiovascular diseases (CVD), such as acute myocardial infarction (AMI) and atherosclerosis, in preclinical studies. Moreover, PTX3 has emerged as novel diagnostic and prognostic biomarker in CVD, reflecting the inflammatory involvement of the vascular bed. In AMI patients, PTX3 levels peak 6-8 hours after the onset of symptoms and in this context it balances pro-inflammatory and anti-inflammatory stimuli.

Since single nucleotide polymorphisms (SNPs) and microRNAs represent two ways in which gene expression can be modified/regulated, we addressed their role in the modulation of PTX3 expression.

Given that *PTX3* gene SNPs have been reported to be associated to different clinical conditions, in particular in innate resistance to infections, we assessed the role of *PTX3* SNPs in affecting PTX3 plasma levels and CVD susceptibility. Despite the characterized role of PTX3 in inflammation, a regulation of PTX3 by microRNAs, which are fundamental fine-tuners of this process, has not yet been described. Therefore, we investigated the role of microRNAs in regulating PTX3 expression.

To our first aim, we performed a candidate-gene association study on Caucasian subjects, in about 1500 healthy individuals and 1700 AMI patients. PTX3 plasma levels were measured by ELISA in healthy subjects as well as in AMI patients from GISSI-Prevenzione trial. Blood was collected from AMI patients at least 5 days after the last event. A significant difference in PTX3 levels was detected between AMI patients and controls, suggesting the persistence of high PTX3 plasma levels in AMI patients up to three months after the last event. Moreover, in AMI patients, PTX3

plasma levels significantly correlated with mortality, but not with cardiovascular death or reinfarction, confirming the prognostic value of this parameter previously described as an independent predictor of 3-months mortality in this pathological condition. Moreover, we report that the 3 *PTX3* SNPs analysed (the intronic rs2305619 and rs1840680 and the exonic rs3816527), alone or combined in haplotypes, are associated with different PTX3 plasma levels. However, we did not find a correlation between the 3 SNPs analysed and the clinical condition of the subject.

About our second aim, data reported in this thesis reveal the existence of a complex network of microRNAs able to down-regulate the basal as well as the TNFα- and IL-1β-induced PTX3 production. The effect of microRNA over-expression was evaluated through the transfection of synthetic pre-miR in the human 8387 fibrosarcoma cell line, able to produce constitutively PTX3. The direct interaction miRNA:mRNA was evaluated through a luciferase reporter assay. Our results reveal that specific microRNAs, like miR-9 and miR-29, directly target and regulate PTX3 mRNA. Other microRNAs, including miR-29, impair PTX3 expression acting on molecules of the signalling pathway that leads to *PTX3* transcription. Among these miRNAs there are also miR-146a and miR-155, two of the major microRNAs involved in controlling the inflammatory response. Another microRNA, miR-181c, impairs PTX3 production by targeting key molecules involved in PTX3 induction and by directly acting on the messenger of ERp18, a molecule involved in PTX3 folding.

In conclusion, our results underline the complexity of PTX3 regulation, revealing that both *PTX3* SNPs and microRNAs are fundamental players of this process.

Introduction

1- The pentraxin superfamily

Pentraxins are pivotal components of the innate immune system that is a first line of resistance against pathogens. Innate immunity plays also a key role in the activation and orientation of adaptive immunity and in the maintenance of tissue integrity and repair[1].

Pentraxins form a superfamily of acute phase proteins with a distinctive multimeric organization. On the basis of the primary structure of the composing protomers, pentraxins are divided into two groups: short and long pentraxins. C-reactive protein (CRP) and serum amyloid P (SAP) components are typical short pentraxins and they represent the main acute phase reactants in human and mouse, respectively[2]. PTX3 is the prototypic long pentraxin, a subfamily of pentraxins that are characterized by the presence of a long N-terminal domain, unrelated to other known proteins, associated with a C-terminal pentraxin-like domain. This last domain is 200 amino acid-long and it contains the conserved 8 amino acid-long pentraxin signature (HxCxS/TWxS, where x is any amino acid) (Fig. 1.1)[3].

Moreover, pentraxins are conserved in evolution from arachnids and insects to humans. Their conservation is testimony to their role in complex organisms. Structural analysis and genetically modified mice have provided a new level of understanding of the role of pentraxins in immunity and homeostasis. Unlike the classic short pentraxins CRP and SAP, whose sequence and regulation have diverged between mouse and human, PTX3 is highly conserved in human and mouse. Thus, results obtained using genetic approaches in the mouse are likely to be informative for the function of PTX3 in human[1], whereas extrapolation from animals to human is more difficult for CRP and SAP[4].

Current literature suggests that short pentraxins are involved in the innate resistance to microbes and participate in the scavenging of cellular debris. On the other hand, studies performed on Ptx3-null mice have shown that this molecule has several functions *in vivo*. PTX3 acts as a soluble pattern recognition receptor (PRR) with a non-redundant protective role against selected pathogens and plays additional

roles at the crossroad between innate immunity, extracellular matrix (ECM) deposition and vascular biology[2].

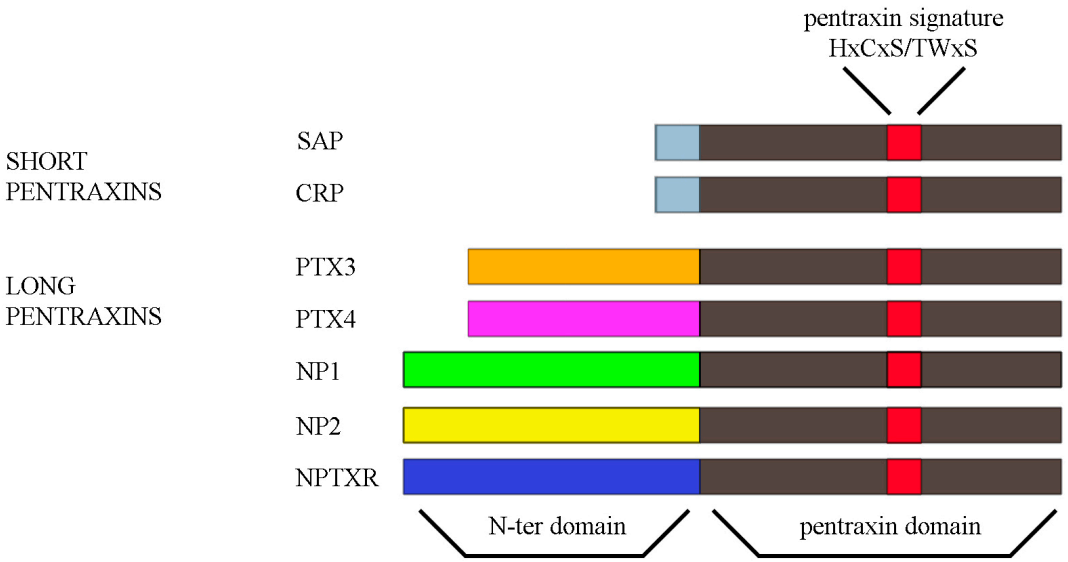

Figure 1.1 | Short and long pentraxin structure. CRP and SAP belong to the short pentraxin subfamily and, like all pentraxins, are characterized by the presence of the pentraxin signature (HxCxS/TWxS, where x is any amino acid) in the 200 amino acid-long C-terminal domain. The N-terminal domain, typical of long pentraxins, like PTX3, is not conserved among the members of this subfamily. Adapted from Garlanda *et al.*[1].

1.1 Short pentraxins

The first pentraxin described was CRP, identified in human serum in the 1930s as the prototypic acute-phase response protein[1,5]. Human SAP was subsequently identified as a relative of CRP on the basis of amino acid sequence identity (51%)[6]. CRP and SAP orthologs in different mammal species share substantial sequence similarity; notable differences include serum basal levels and changes during the acute-phase response, with CRP and SAP being the main acute-phase reactants in human and mouse, respectively.

1.1.1 Gene organization and expression

The *CRP* gene is located on chromosome 1q23 and is organized in two exons, the first coding for the leader peptide and for the first two amino acids of the mature protein and the second coding for the remaining 204 amino acids. CRP levels in the plasma of healthy adults are barely detectable (≤ 3 mg/L), but they increase as much as 1000-fold following an acute-phase stimulus as a result of accelerated rates of

transcription in the liver, mainly in response to the proinflammatory cytokine IL-6[7]. Expression of CRP mRNA in tissues other than the liver has also been reported, but apparently extra hepatic sources do not contribute to CRP plasma levels. CRP has been extensively used clinically for over 75 years as a nonspecific systemic marker of infection, inflammation, and tissue damage.

SAP is produced exclusively by hepatocytes and is the main acute-phase protein in mice, whereas in human serum it is constitutively present at 30 to 50 mg/L.

1.1.2 Protein structure

Human CRP is composed of five identical non-glycosylated protomers with a total molecular weight of 115 kDa. The amino acid sequence includes two cysteines in positions 36 and 97 conserved in all the members of the pentraxin family and involved in intra-chain disulfide bonds[8].

SAP is a glycoprotein made up of five non-covalently attached subunits of 23 kDa each.

1.1.3 Ligand specificity, receptors and functions

The physiological functions attributed to pentraxins involve recognition and binding to different ligands, mostly in a Ca^{2+}-dependent manner. The relationship between ligand binding and function of pentraxins is a matter of debate. A list of selected ligands recognized by CRP and SAP is provided in Table 1.1[9].

Table 1.1 | Main cellular sources and ligands of short pentraxins. Adapted from Bottazzi *et al.*[50].

Short pentraxins (CRP, SAP)	
Main cellular sources	**Ligands**
Liver (hepatocytes)	- Complement components (C1q, Factor-H, L-ficolin, M-ficolin) - Microorganisms (bacteria, viruses, fungi, parasites) - Phosphorylcoline, carbohydrates - Modified LDLs - ECM protein (fibronectin, collagen IV, laminin, proteoglycans) - Amyloid fibrils - DNA

The first ligand described for CRP was the C-polysaccharide of *Streptococcus pneumoniae*. This interaction is due to a direct binding of CRP to phosphorylcholine (PC), a major constituent of C-type capsule polysaccharides. Moreover, CRP binds various microorganisms, including fungi, yeasts, bacteria, and parasites (*Plasmodium, Leishmania*), through PC and carbohydrate structures, promoting phagocytosis and resistance to infections[10]. Binding to bacteria is not always necessary for protection because CRP also protects mice from infection with *Salmonella typhimurium*, a pathogen to which CRP does not bind[11]. Furthermore, CRP-mediated activation of the classical complement pathway (see below) has no role in protecting mice against *S. pneumoniae* infection[12].

Like CRP, SAP binds various bacteria, such as *Streptococcus pyogenes* and *Neisseria meningitidis*[13]. Moreover, binding to influenza virus has also been reported[14]. SAP binds to several bacteria via lipopolysaccharide (LPS) and prevents LPS-mediated complement activation and LPS toxicity[13,15]. However, for certain organisms to which SAP binds, such as *S. pyogenes* and rough strains of *Escherichia coli*, SAP enhances virulence by protecting the bacteria against phagocytosis, whereas it is protective in infection with organisms to which it does not bind, for instance *Listeria monocytogenes*[13].

CRP binds to modified low-density lipoprotein (LDL) via the PC and cholesterol moieties present on LDL[16] and co-localizes with LDL in human atherosclerotic lesions[17]. However, CRP is neither atheroprotective nor proatherogenic in mouse models of human atherosclerosis[4].

Pentraxins recognize damaged cells and their constituents and are implicated in *in vivo* disposal of apoptotic cells. Human CRP binds to the membranes of damaged cells and apoptotic cells during late phases of apoptosis, via various phospholipids (PC and phosphoethanolamine) and small nuclear ribonucleoprotein particles and chromatin/nucleolar components that redistributes to the plasma membrane during late apoptosis[7]. Human SAP binds *in vivo* to chromatin[18].

CRP and SAP, aggregated or attached to most of their ligands, interact with the globular head of C1q, the recognition subunit of the classical complement pathway, and activate the classical complement cascade[19]. The three-dimensional structure of CRP shows the presence of a deep, extended cleft in each protomer on the face of the pentamer opposite that containing the PC-binding sites. Mutational analysis of

residues participating in the formation of this pocket identified the amino acids involved in C1q binding and complement activation[20].

Complement activation by short pentraxins may be one of the mechanisms leading to the removal of cellular debris[21]. When bound to self surfaces (e.g., apoptotic cells, damaged tissue), CRP activates the classical pathway of complement through interaction with C1q, but this activation is restricted to the initial stages with only little consumption of C5-C9[22]. Furthermore, surface-bound CRP inhibits the alternative pathway amplification loop, possibly through the interaction with Factor H, the main soluble regulator of the alternative pathway of complement activation[23]. However, it is currently unclear whether this is a physiological phenomenon[24]. SAP binds to matrix components such as laminin, type IV collagen, fibronectin, and proteoglycans. Moreover, SAP binds to β-amyloid peptide in a Ca^{2+}-dependent manner and contributes to the pathogenesis of amyloidosis[25].

A specific and saturable binding to all three classes of Fcγ receptors (FcγR) has been demonstrated for both CRP and SAP, and the interaction with FcγR is able to mediate phagocytosis of apoptotic cells and microorganisms, as well as mediate protective immune responses[26,27]. However, the interpretation of these data has been questioned[28]. The crystal structure of human SAP in complex with the extracellular domain of FcγRIIa has recently been characterized, indicating that the contact region between the two molecules involves the ridge helices of pentameric SAP. The functional activation of FcγR-mediated phagocytosis and cytokine secretion have been described. The stoichiometry of the interaction infers the requirement for multivalent pathogen binding for receptor aggregation[29]. These studies show that SAP and IgG share the binding site and compete for FcγR binding and that pentraxins inhibit immune complex–mediated phagocytosis.

Thus, pentraxins possess similar functions to those of antibodies that activate both the complement and FcγR pathways.

1.1.4 CRP plasma levels, the risk of coronary heart disease (CHD) and *CRP* genetic variations

CRP is a nonspecific systemic marker of infection, inflammation, tissue damage and/or almost any form of adverse non-physiological stress. For this reason, an increased CRP concentration might reflect any of a very wide range of serious diseases which have not yet declared themselves, for example, Hodgkin's disease or

renal carcinoma or any other condition which is often occult for some time before clinical presentation[4].

In healthy subjects CRP plasma concentration is usually within the range 0.1-3 mg/L. However, after a strong acute stimulus (e.g. sepsis or AMI), it can rise by over 1000-fold in 24-48 h and fall with a half time of about 24 h when the stimulus is removed[4]. Several population-based prospective studies of coronary heart disease (CHD) have reported an association between baseline CRP levels and CHD risk[30,31]. These observations, together with the reported ability of CRP to bind LDL[16] and its presence in the atherosclerotic plaque[17], have collectively raised the possibility that CRP may play a direct causal role in CHD (and, by implication, could be an important therapeutic target), although there is debate about the interpretation of both epidemiological and experimental observations[32-34].

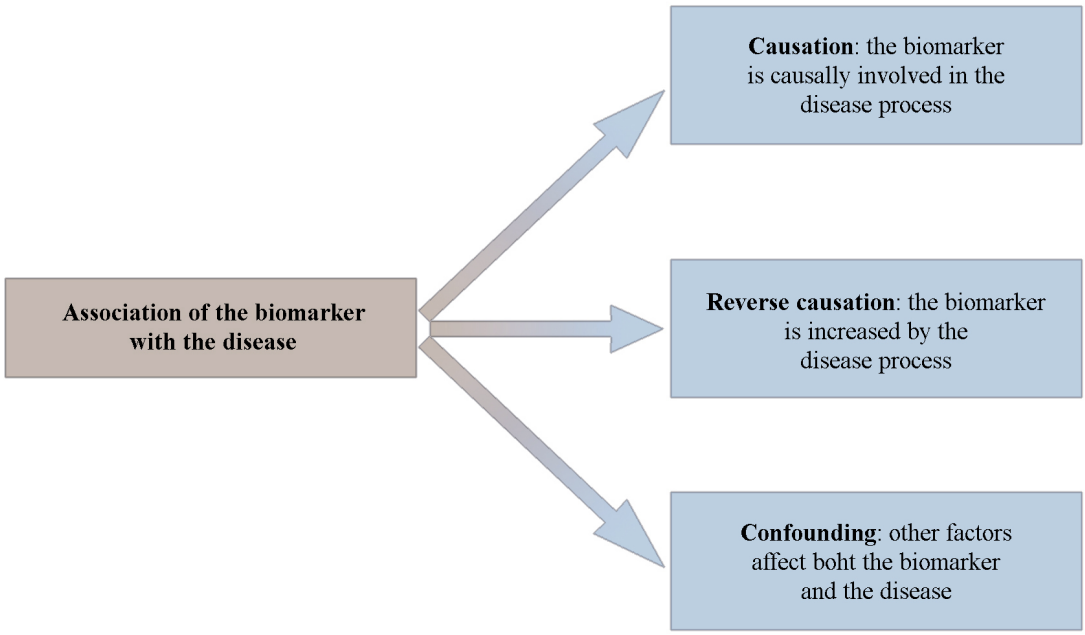

Figure 1.2 | Types of association of a biomarker with a disease. The association between a biomarker (e.g., CRP) and a disease (e.g., atherosclerosis) may be represented by a causal relationship (causation: an increase in the biomarker as a consequence of the disease), by a reverse causation (the disease itself determines an increase of the biomarker), or by an association that is spurious because both the biomarker and the disease are affected independently by another known or unknown factor (confounding). Adapted from Schunkert *et. al.*[38].

The statistical strength of the association between CRP levels and CHD risk, derived from epidemiological studies, is at least as robust as that of established risk factors such as hypertension, diabetes mellitus, and hyper-cholesterolemia[35].

However, statistical strength does not imply causality, since confounding factors or reverse causality offer alternative explanations for the association (Fig. 1.2). CRP is particularly susceptible to confounding, since multiple cardiovascular risk factors, including smoking, hypertension, obesity, lack of physical activity, and low socioeconomic status, all relate independently to elevated plasma levels of the protein[36,37]. Reverse causation is also a potential explanation, since atherosclerosis may trigger an elevation of CRP levels[38].

Since randomized trials of interventions, specific to CRP, have not yet been done in relation to cardiovascular disease outcomes, till now causality has been judged only with focused genetic studies[39].

Several single nucleotide polymorphisms (SNPs) within the *CRP* gene have been identified and four of them influence its circulating concentration[40]. *CRP* genotypes (and haplotypes) are clearly associated with CRP levels, but not with any of a large panel of established or emerging cardiovascular risk markers[41]. Indeed, *CRP* genetic variants responsible for changes in CRP plasma levels were used as an instrument for testing causality of CRP in atherosclerosis[42]. For doing that, scientists took advantage of the fact that persons are effectively randomly assigned at birth to either higher or lower CRP levels depending on the genetic variant they received from their parents. According to Mendel's law of independent assortment, neither endogenous nor exogenous factors disturb this randomization process, which therefore can be termed "Mendelian randomization"[43]. This generalization is not entirely free of exceptions, since genes that are very close to one another on a chromosome do not segregate independently; however, it is probably true for most traits (Fig. 1.3)[38]. Once the quantitative association between CRP and CHD risk together with the quantitative correlation between *CRP* SNPs and CRP concentration were determined, scientists could predict the expected effect of the genetic variants on ischemic events. The observed association was markedly different from the predicted effect: none of the *CRP* variants were associated individually or in combination with the risk of ischemic events (Fig. 1.4)[38,39,42]. Taken together these data indicate that CRP concentration itself is unlikely to be even a modest causal factor in CHD[39].

Irrespective of the causal relevance of C reactive protein itself to CHD, however, there is considerable evidence that persistent inflammation might contribute to CHD[39]. Actually, the results of the large multicenter trial JUPITER have shown a remarkable reduction of cardiac events with rosuvastatin when compared to placebo

in about 18000 apparently healthy men and women, with normal LDL (<130 mg/dL) but CRP>2mg/L[44]. Though this is not a definite proof of the etiological role of inflammatory activation in cardiac disease, it extends the indication for preventive treatment aimed at decreasing CRP levels to a large class of subjects for whom there was not any indication in the past for a statin.

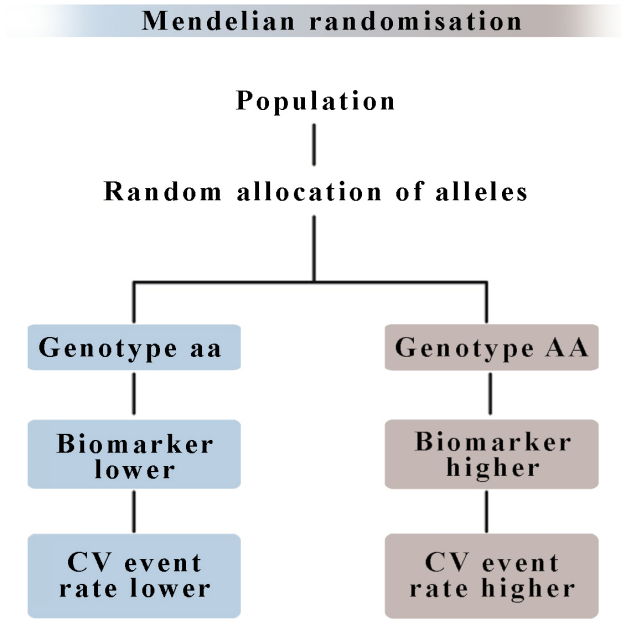

Figure 1.3 | Conceptual diagram of a genetic "Mendelian randomization" experiment to judge the causal relevance of a biomarker associated with CHD risk. Adapted from Casas *et al.*[4].

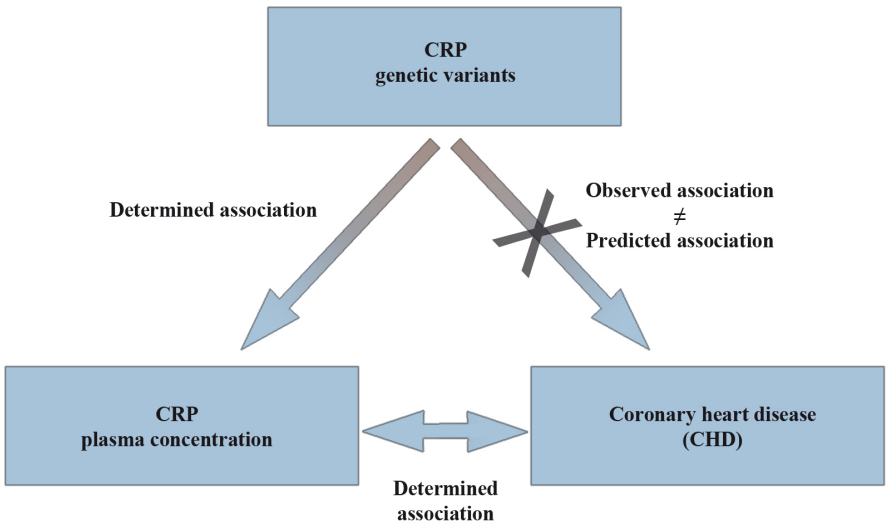

Figure 1.4 | "Mendelain randomization" approach used for identifying the causal association between CRP and CHD. The mendelian randomization approach allows determination of a causal relationship between a biomarker and disease. It starts with the evidence that the biomarker is

associated with the disease (lower line of triangle). The quantitative nature of this association is determined. Genetic variants are then identified that affect the level of the biomarker (left line of triangle), and the quantitative nature of this relationship is also determined. Combining these two sets of information allows prediction of the effect of the genetic variant on disease risk if the association of the biomarker with the disease is causal. This prediction is then tested (right line of the triangle). If the predicted association of the variant with the disease is observed, then this would strongly suggest a causal relationship between the biomarker and the disease (since the relationship between the genetic variant and the disease can go in only one direction). If, on the other hand, no association is seen between the genetic variant and the disease, as observed for CRP, this would indicate that the association between the biomarker and the disease is due to either reverse causation or confounding. Adapted from Schunkert *et. al.*[38].

Is CRP mainly a causal risk factor in CHD, mainly a marker of established cardiovascular risk factors to which it is correlated, or mainly a marker of subclinical disease or some combination of these possibilities? This is an open question. A completely distinct possibility is that, in contrast to modest long-term differences in baseline CRP values, the major acute phase response of CRP triggered by AMI may contribute acutely to severity and outcome of the ischaemic lesion[45].

1.2 Long pentraxins

PTX3 is the first member of the long pentraxin subfamily to be identified in the early 1990s as a new secreted protein rapidly induced by IL-1β in endothelial cells (ECs) or by TNFα in fibroblasts[46,47].

The long pentraxins identified after PTX3 include guinea pig apexin, neuronal pentraxin (NP or NPTX) 1 and NP2, also called NPTX2 or NARP, and NPTX receptor, which is the only member associated to the cell through a trans-membrane domain. NPTXs have been shown to be involved in the excitatory synaptic remodeling. NPTX2 has been implicated in long-term neuronal plasticity as well as dopaminergic nerve cell death and NPTX1 in hypoxia-ischemia and amyloid-b–induced neuronal death. Recently, a search for pentraxin domain-containing sequences in different databases resulted in the identification of PTX4, a new long pentraxin, that, conserved among mammals, is also present in *Xenopus laevis* and zebrafish. PTX4 has a unique pattern of mRNA expression. For instance, its expression is high in the stroma of thymus and spleen and low in the brain and it does not behave as an acute phase gene[48].

1.2 PTX3

1.2.1 Gene organization and expression

The human *PTX3* gene (NCBI accession number: X7748; GI: 1679604) has been mapped on chromosome 3 (3q25) and is organized in three exons separated by two introns. The first two exons code for the leader peptide and the N-terminal domain of the protein and the third exon encodes the pentraxin domain. The murine gene presents the same structural organization and is located on chromosome 3. The *PTX3* gene shows high evolutionary conservation in sequence, gene organization and regulation, making it possible to address its pathophysiological roles in genetically modified mice. The proximal promoters of both human and murine *PTX3* genes share numerous potential transcription factor binding sites including Pu1, AP-1, NF-κB, SP1 and NF-IL-6 sites. It has been shown that the NF-κB-binding site is essential for the transcriptional response to pro-inflammatory cytokines (e.g. TNFα and IL-1β), even if the SP1 site seems to have a role in this inducibility. On the other hand, AP-1 controls the basal transcription of PTX3[49].

PTX3 was originally identified as a cytokine-inducible gene in vascular endothelial cells (ECs) and fibroblasts. PTX3 expression is rapidly induced in a variety of additional cell types by several stimuli such as cytokines (e.g. TNFα and IL-1β), toll-like receptor (TLR) agonists, microbial moieties (e.g. LPS, outer membrane protein A, OmpA, and lipoarabinomannans) or intact microorganisms (Fig. 1.5). Beside IL-1β and LPS, oxidized and enzymatically modified low-density lipoproteins (ox-LDL) promote PTX3 production in ECs and human primary vascular smooth muscle cells (SMCs). Similar inflammatory signals induce PTX3 expression in other cell types including myeloid dendritic cells (DCs), macrophages, kidney epithelial cells, synovial cells, chondrocytes, adipocytes, alveolar epithelial cells, glial cells, mesangial cells and granulosa cells[50,51]. PTX3 is constitutively stored in the specific granules of neutrophils and is released in response to TLR engagement by microorganisms or TLR agonists[52,53]. Expression of the PTX3 mRNA is temporally confined to immature myeloid cells. PTX3 can partially localize in neutrophil extracellular traps (NETs) formed by extruded DNA. In this context the neutrophil-associated PTX3 promotes the generation of an antimicrobial micro-environment that is essential for trapping and killing microbes[52]. In contrast to these cell types, which express PTX3 upon stimulation, both human and murine lymphatic ECs constitutively

express the protein[54]. Resting T and B lymphocytes and natural killer cells do not express PTX3 mRNA.

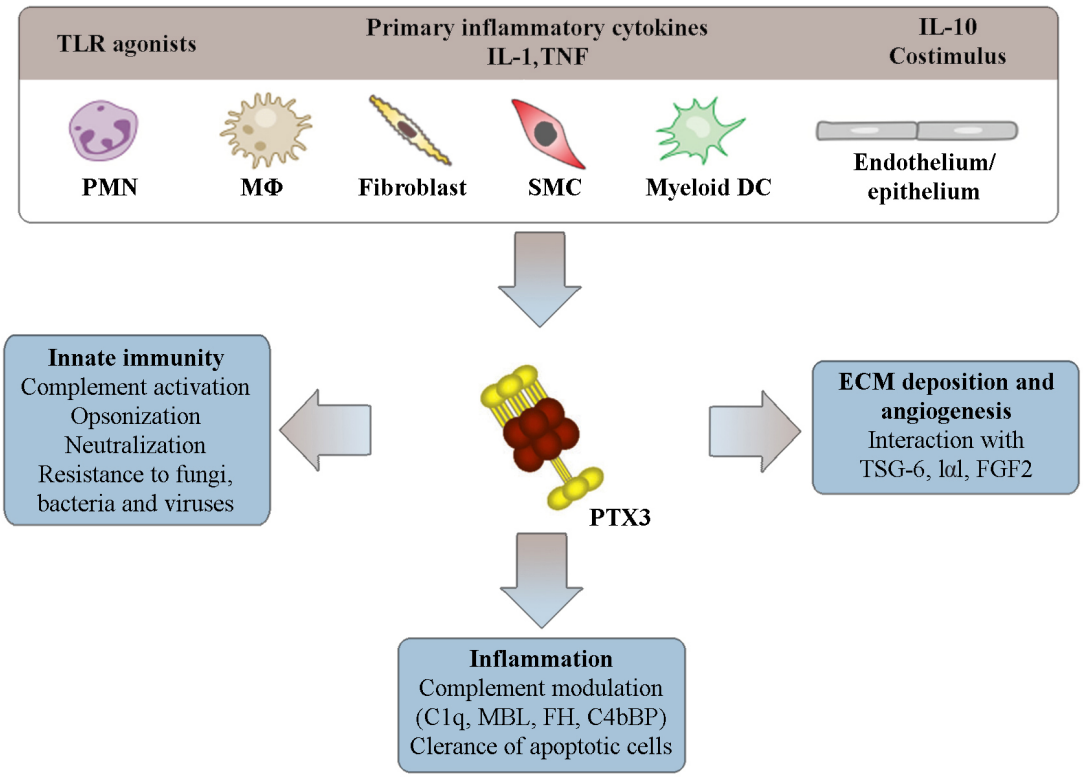

Figure 1.5 | Cellular sources and functions of the long pentraxin PTX3. PTX3 is produced by phagocytes (PMN, MΦ, myeloid DC), fibroblasts, SMCs, ECs and, to a lesser extent, by epithelial and mesenchymal cells, upon stimulation with microbial moieties (TLR agonists) or primary inflammatory cytokines (IL-1, TNF); IL-10 acts as a costimulus for proinflammatory stimuli. Once released, PTX3 participates (i) in innate immune responses to fungi, bacteria, and viruses, through complement activation and regulation, microorganism opsonization, viral neutralization; (ii) in inflammatory responses, by modulating complement activation, by participating in the clearance of apoptotic cells, and possibly by other undefined mechanisms. PTX3 also participates in extracellular matrix deposition and angiogenesis. Adapted from Bottazzi *et al.*[50].

Interferon-γ inhibits PTX3 production in DCs, monocytes and macrophages by reducing both gene transcription and transcript stability, while IL-10 amplifies LPS-induced PTX3 expression[53,55]. IL-4, dexamethasone, vitamin D3 and prostaglandin E2 also inhibit LPS-induced PTX3 in myeloid DCs[55].

Different signalling pathways can affect PTX3 production, depending on cell type and/or stimuli. PTX3 expression in a model of acute myocardial ischaemia is controlled by the NF-κB pathway[56], while induction of the protein by TNFα in lung epithelial cells does not require NF-κB, but rather involves the c-Jun N-terminal

kinase pathway[57]. Moreover, production of PTX3 in ECs that is induced by high-density lipoproteins (HDL) requires the activation of the PI3K/Akt pathway through G-coupled lysosphingolipid receptors[58].

Glucocorticoid (GC) hormones induce or enhance PTX3 production in non-haematopoietic cells (e.g. fibroblasts and ECs), but inhibit PTX3 production in haematopoietic cells (e.g. DCs and macrophages)[59]. These divergent effects are likely due to the fact that in non-haematopoietic cells, the GC receptor acts as a transcription factor, whereas in haematopoietic cells it interferes with the action of other signalling pathways, probably NF-κB and AP-1.

1.2.2 Protein structure

Human PTX3 is a multimeric glycoprotein, whose composing subunits are made of 381 amino acids, including a 17-residue signal peptide. PTX3 primary sequence is highly conserved among animal species. Like other members of the long pentraxin family, PTX3 is composed of a unique N-terminal region coupled to a 203-amino acid C-terminal domain homologous to the short pentraxins CRP and SAP[60] (Fig. 1.1). The N-terminal region (residues 18–178 of the pre-protein) is unrelated to any known protein structure. Nevertheless, secondary structure predictions indicate that this part of the protein is likely to form four α-helices, three of which (amino acids 78–97, 109–135 and 144–170) are probably involved in the formation of coiled-coil structures[51]. As stated above, the C-terminal domain of PTX3 (residues 179–381 of the pre-protein) is homologous to the short pentraxins CRP and SAP (57% similarity)[60]. Therefore, three-dimensional models of this domain have been generated that are based on the crystallographic structures of CRP and SAP and show the pentraxin domain of PTX3 to adopt a β-jelly roll topology, similar to that found in legume lectins (Fig. 1.6). A single N -glycosylation site has been identified in the C-terminal domain of PTX3 at Asn220[61]. This site is fully occupied by complex-type oligosaccharides, mainly fucosylated and sialylated biantennary sugars with a minor fraction of tri- and tetrantennary glycans. Most importantly, PTX3 glycosylation has been shown to affect the protein binding to a number of ligands, thus suggesting that changes in the glycosylation status might represent a strategy to tune the biological activity of this long pentraxin (Fig. 1.7).

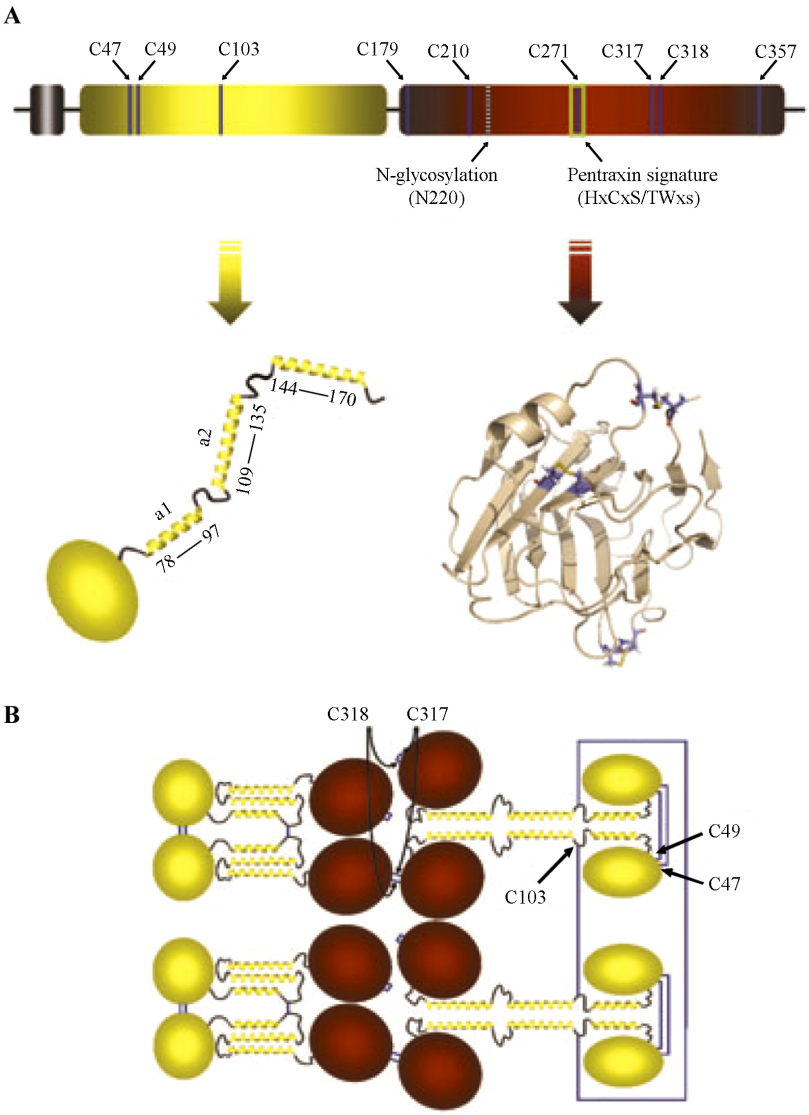

Figure 1.6 | Model of PTX3 octamer: arrangement of protomer subunits. (A) Schematic representation of the PTX3 protomer showing the N-terminal domain in yellow, followed by the globular pentraxin domain in red. Positions of the Cys residues, the N-glycosylation site at Asn220 and the pentraxin signature motif are indicated. Arrows point to a topological drawing of the N-terminal domain, which is believed to be composed of a globular region and three α-helical segments (α1, α2 and α3), and a three-dimensional model of the C-terminal pentraxin domain based on the crystal structure of C-reactive protein (Protein Data Bank ID: 1b09). **(B)** Disulfide bond organization of the PTX3 octamer. The α-helical regions of the N-terminal domains in yellow are predicted to form coiled-coil-like structures and hypothesized to adopt two distinct structural arrangements: either an extended conformation (right) in which four protomers associate through inter-chain interactions (i.e. to form tetramers) or a compact organization (left) where each protomer self-associates to form an antiparallel three-helix bundle (i.e. to form dimer of dimers). Cys317 and Cys318 in the C-terminal pentraxin domains (denoted in red) link the protomers (i.e. a tetramer and two dimers) into octamers. Adapted from Inforzato *et al.*[2].

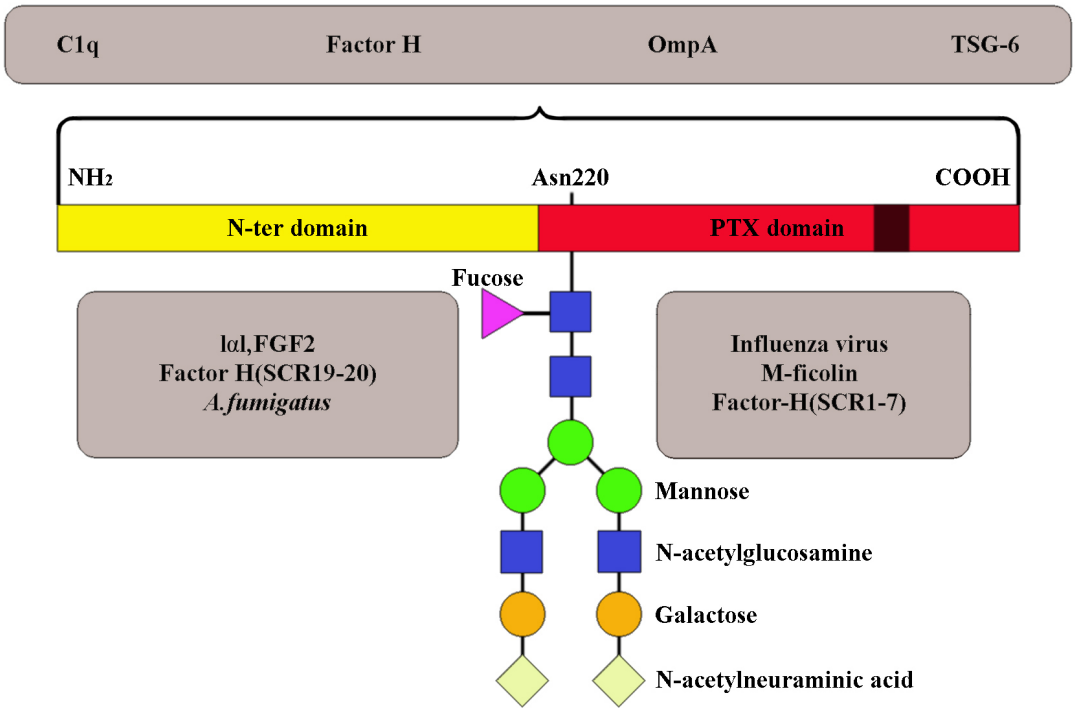

Figure 1.7 | Role of the two PTX3 domains in recognizing ligands. The overall molecule is necessary for optimal interaction with complement components (C1q and Factor H), microbial moieties (OmpA), and the extracellular matrix protein TSG-6. Recombinant N-ter domain can reconstitute the full-length protein in the interaction with IαI and FGF2. Interaction with conidia of *A. fumigatus* is also mediated by N-ter domain, whereas influenza virus and M-ficolin interact with the sialic acid present on the glycosidic moiety at Asn220, localized in the PTX domain. Adapted from Bottazzi *et al.*[50].

In addition to the multidomain organization, the human PTX3 protein shows a complex quaternary structure with eight protomer subunits held together by both covalent and non-covalent interactions[60]. In this regard, mass spectrometry and site-directed mutagenesis analyses of the recombinant human protein indicate that cysteine residues at positions 47, 49 and 103 in the N-terminal region form three inter-chain disulfides holding four protein subunits in a tetrameric arrangement. Two tetramers are linked together to form an octamer by additional inter-chain bridges involving the C-terminal domain cysteines Cys317 and Cys318 (Fig. 1.6). A low-resolution model of the intact PTX3 molecule has been generated based on the data from electron microscopy and small-angle X-ray scattering (SAXS) that show that eight subunits of the protein fold into an elongated structure with a large and a small domain interconnected by a stalk region[62]. This oligomerization state and the asymmetric shape of the molecule make PTX3 unique among pentraxins. The only other pentraxin that forms an octamer is SAP from *Limulus polyphemus*, which has been reported to fold into a doubly stacked octameric ring.

As anticipated above, the structural determinants of the PTX3 quaternary organization are mainly localized in the N-terminal domain, where this region mediates the association of protomers into tetramers via both covalent (i.e. disulfide bonds) and non-covalent (i.e. inter-chain coiled coils) interactions[62]. Besides their structural role as building blocks of the PTX3 molecule, the N-terminal domain tetramers act as functional units in the recognition of PTX3 ligands. Therefore, the PTX3 quaternary structure plays a key role in dictating the protein-binding properties and, ultimately, its biological functions.

1.2.3 Ligand specificity and receptors

The physiological functions attributed to pentraxins involve recognition and binding to different ligands, including microbial moieties, complement components, P-selectin and ECM proteins (Table 1.2).

Similarly to short pentraxins, PTX3 binds a number of selected bacteria, fungi and virus: a specific binding has been observed to conidia of *Aspergillus fumigatus*, *Paracoccidioides brasiliensis* and zymosan, to selected Gram-positive and Gram-negative bacteria and finally to some viruses such as human and murine cytomegalovirus and influenza virus type A (IVA). The interaction between PTX3 and IVA occurs through binding of sialylated oligosaccharides on PTX3 to the viral

Table 1.2 | Main cellular sources and ligands of the long pentraxin PTX3. Adapted from Bottazzi *et al.*[50].

PTX3	
Main cellular sources	**Ligands**
Monocytes, MØ, PMN, EC, DC, fibroblasts, epithelial cells	- Complement components (C1q, Factor-H, C4BP, L-ficolin, M-ficolin, MBL) - Microorganisms (bacteria, viruses, fungi) and microbial moieties (OmpA) - ECM protein (IαI, TSG-6) - Apoptotic cells - FGF2

haemagglutinin and results in the neutralization of virus infectivity *in vitro*[63]. Consistently, desialylated PTX3 does not bind IVA and does not neutralize virus infectivity. In addition to viral haemagglutinin, other microbial ligands have been identified. PTX3 binds OmpA from *Klebsiella pneumoniae* and amplifies the TLR2-

dependent inflammatory response induced by OmpA after recognition of scavenger receptors LOX-1 and SREC-I. Pre-treatment with complement inhibitors abrogates PTX3 enhancement of OmpA pro-inflammatory effect, underlining the interplay among the cellular and the humoral arms of innate immunity in recognizing microbial moieties[64].

The interaction of PTX3 with the complement system has been known since the first characterization of the protein. PTX3 recognizes and modulates the activity of key components of all three-complement pathways (i.e. C1q, ficolins, mannose-binding lectin (MBL), Factor H and C4b-binding protein (C4BP)]. PTX3 binds the first component of the classical complement cascade, C1q, interacting with the C1q globular head[60]. The interaction of PTX3 with plastic-immobilized C1q, an experimental condition that mimics the surface of microbes, results in the activation of the classical complement pathway. However, PTX3 inhibits the C1q haemolytic activity when the interaction occurs in the fluid phase, probably due to competitive blocking of relevant sites[65]. In addition, like CRP, PTX3 interacts with factor H, the main soluble regulator of the alternative pathway of complement, thus promoting factor H deposition on PTX3-coated surfaces and preventing exaggerated complement activation[66]. Moreover, PTX3 interacts with C4BP, a classical/lectin complement pathway inhibitor, enhancing its binding to apoptotic cells and recruiting functionally active C4BP to human fibroblast- and endothelial cell-derived extracellular matrices *in vitro*. This interaction probably prevents excessive local complement activation[67]. PTX3 also interacts with ficolin-2[68] and MBL[69], thus enhancing complement deposition on *A. fumigatus* conidia and opsono-phagocytosis of *Candida albicans* by polymorphonuclear leucocytes, respectively. Finally, PTX3 binds M-ficolin and immobilized PTX3 is able to trigger M-ficolin–dependent activation of the lectin complement pathway[70].

Assessing the capacity of PTX3 to bind secreted cytokines and growth factors, it was found that PTX3 has a significant interaction with fibroblast growth factor 2 (FGF2): PTX3 sequesters the growth factor in an inactive form, acting as a 'FGF2 decoy' and tuning the neovascularization process[71].

PTX3 also interacts with ECM proteins such as TNFα-induced protein 6 (TSG-6) and inter-α-trypsin inhibitor (IαI). The binding of PTX3 to TSG-6 and IαI is essential for the correct organization of the viscoelastic matrix of cumulus oophorus and the

lack of PTX3 is associated with female subfertility as a consequence of cumulus matrix instability[72,73].

Although most of the ligands recognized by PTX3 require the full-length protein for optimal binding, the sites of interaction with both IαI and FGF2 are entirely localized in the PTX3 N-terminal domain[51,73]; this region of the protein is also responsible for the interaction of PTX3 with *A. fumigatus* conidia and factor H short consensus repeats 19–20[66,74] (Fig. 1.7). Recombinant preparations of the N-terminal domain are endowed with the same functions as the full-length PTX3 with regard to organization of cumulus oophorus matrix and inhibition of FGF2-dependent proliferation of ECs[51,73].

The observation that macrophages display a binding site for PTX3 suggested the existence of a cellular receptor for this protein[75]. In the context of a study focused on SAP, Lu *et al.*[29] recently reported that PTX3, similarly to CRP and SAP, can interact with Fcγ receptor (FcγR), in particular FcγRIII/CD16 and FcγRII/CD32, with K_d 1.6 and 18.7 μM, respectively. In a parallel investigation, we observed a key role of CD32 in the opsonic activity of PTX3 towards *A. fumigatus*[74]. It remains to be established whether PTX3 receptors other than FcγR exist.

1.2.4 PTX3 function in innate immunity

As stated above, PTX3 can interact with a number of different pathogens including selected fungi, bacteria and viruses. According to the binding properties, Ptx3-deficient mice are more susceptible than wild-type mice to infections caused by these pathogens. In particular, Ptx3-deficient animals are susceptible to *A. fumigatus* infection as a result of a defective recognition of conidia by alveolar macrophages and DCs. This susceptibility is associated with a low-protective T helper 1 (Th1) antifungal response coupled with an inappropriate Th2 response. Treatment with recombinant PTX3 restores the protective Th1 response showing that PTX3 can participate in the tuning of immune responses[75]. PTX3 interacts also with zymosan, a complex carbohydrate of the yeast cell wall that induces inflammatory signals in macrophages upon recognition by dectin-1, TLR2 and TLR6. PTX3 binding to zymosan promotes clearance of zymosan particles and zymosan-containing pathogens (i.e. *P. brasiliensis*) by macrophages in a dectin-1-dependent manner. In accordance, Ptx3-overexpressing mice have an increased phagocytic index towards the fungal

pathogen *P. brasiliensis* compared to wild-type animals and exogenous PTX3 can enhance the phagocytic activity of wild-type macrophages[76].

In the case of *K. pneumoniae* infection, overexpression of PTX3 by transgenic mice during infection was associated with an enhanced ability to produce pro-inflammatory mediators, including nitric oxide (NO) and TNFα, and, as a consequence, with protection or faster lethality, depending on the dose of inocula.[77] It is likely that, through its interaction with *Kp*OmpA, PTX3 might modulate the pro-inflammatory response triggered by *K. pneumoniae*[64].

Recent results indicate that PTX3 has therapeutic activity in chronic lung infections by *Pseudomonas aeruginosa*, which mimic lung infections of cystic fibrosis (CF) patients, by enhancing bacteria clearance, reducing the production of pro-inflammatory cytokines, neutrophil recruitment in the airways and histopathological lesions[78].

Bozza *et al.* found that upon binding to human and murine cytomegalovirus (MCMV), PTX3 reduced viral entry and infectivity in DCs *in vitro*. Consistently, Ptx3-deficient mice are more susceptible to MCMV infection than wild-type mice and PTX3 protects susceptible BALB/c mice from MCMV primary infection and reactivation *in vivo*[79].

Similarly, upon binding to influenza virus (H3N2), PTX3 inhibits virus-induced haemagglutination and viral neuramidase activity and neutralizes the virus infectivity *in vitro* and reduces mortality and the viral load *in vivo* in mice[63]. However, it has been recently reported that both seasonal and pandemic H1N1 influenza A viruses were resistant to the antiviral activity of PTX3[80].

The molecular mechanisms underlying the opsonic activity of PTX3 have been recently investigated: PTX3 enhances the recognition and phagocytosis of conidia by neutrophils through an FcγRII-, CD11b- and complement-dependent mechanism[74].

1.2.5 PTX3 function in inflammation and cardiovascular disease

The *in vivo* role of PTX3 in inflammatory conditions has been investigated using both Ptx3-overexpressing and Ptx3-deficient mice. PTX3 overexpression resulted in increased resistance to sepsis in the caecal ligation and puncture model[81], but in increased mortality and inflammation in intestinal ischaemia and reperfusion injury[82].

In vivo models also showed that PTX3 is involved in modulating inflammation in sterile conditions, for instance, in acute myocardial infarction (AMI) and

atherosclerosis[56,83](Fig. 1.8). Actually, as already mentioned, two of the main cellular components of the atherosclerotic lesion, namely ECs and macrophages, are major producers of PTX3 during inflammation. Accordingly, immunohistochemical staining showed a strong expression of PTX3 in advanced atherosclerotic lesions. PTX3 immunoreactivity was observed in the ECM, ECs and macrophages within the lesion, as well as in subendothelial SMCs, neutrophils and in foam cells within lipid-rich areas of the plaque[83]. This is consistent with the observation that IL-1, LPS and ox-LDL induce PTX3 production in cultured human SMCs.

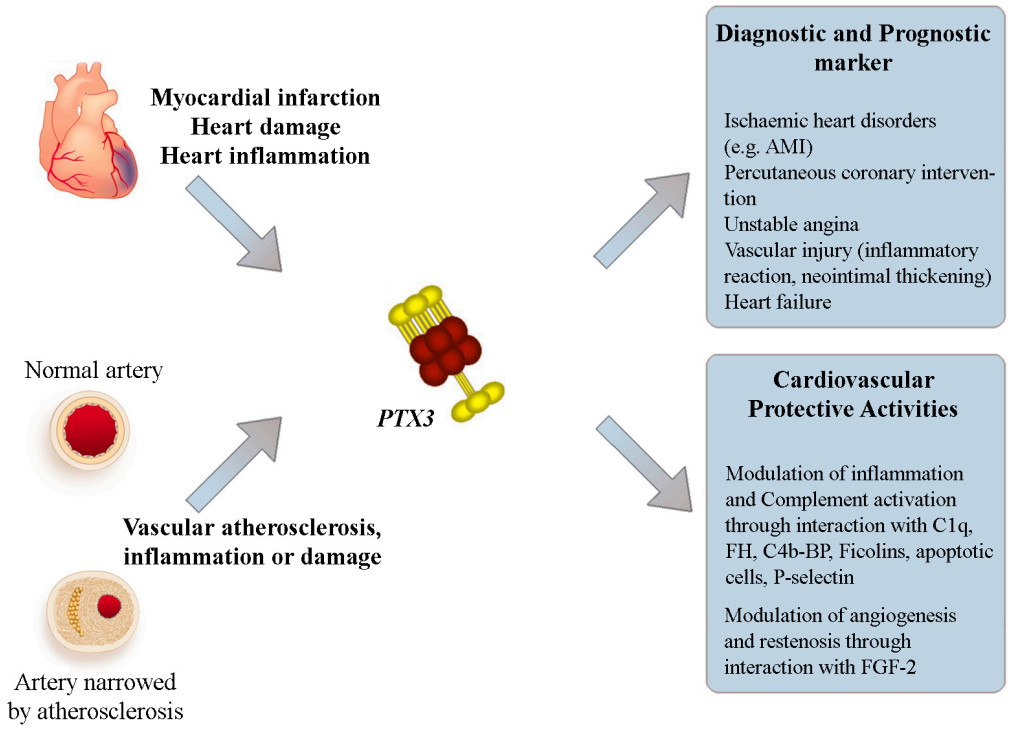

Figure 1.8 | **The role of PTX3 in CVD.** PTX3 is rapidly induced at the tissue level and released in plasma at sites of myocardial infarction, atherosclerosis, vascular damage or inflammation. The released protein interacts with different ligands and participates to the pathogenesis of CVD by modulating inflammation, complement activation, angiogenesis and restenosis. PTX3 also represents a useful diagnostic and prognostic biomarker in cardiovascular pathologies.

In *in vivo* experiments[56], PTX3 was observed to peak in the serum of wild-type mice after coronary artery ligation-induced ischaemia followed by reperfusion, similar to humans, and infarcts were significantly larger in Ptx3-deficient mice than in wild-type littermates. It has been proposed that PTX3 might exert a protective cardiovascular role by modulating complement activation: in this context a higher

deposition of C3 was indeed observed in the infarct areas of Ptx3-deficient mice. Also, the interaction between PTX3 and factor H, which promotes deposition of factor H on PTX3-coated surfaces as discussed above, could represent a way to prevent tissue damage associated to excessive complement activation. Moreover, the finding that PTX3 binds P-selectin and attenuates neutrophil recruitment at sites of inflammation indicates that PTX3 may have in this context a regulatory role on inflammation by acting as a feedback mechanism of inhibition of leukocyte recruitment. Recent observations demonstrate that PTX3 binds to activated circulating platelets in AMI patients, dampening their proinflammatory and prothrombotic action[84]. All these findings suggest that PTX3 is endowed with a potential cardiovascular protective effect (Fig. 1.9), a view that is further supported by the recent observation that PTX3 deficiency enhances atherosclerosis in $ApoE^{-/-}$ mice and increases macrophage accumulation in the atherosclerotic lesions. In this animal model of atherosclerosis, the lack of PTX3 was associated with increased bone marrow monocytosis, which in turn was found to correlate with the extent of the lesion size. Furthermore, mice lacking PTX3 showed an increased expression of adhesion molecules, cytokines and chemokines in the vascular wall, thus suggesting that PTX3 may modulate the vascular-associated inflammatory response. Whether this effect is dependent upon vascular or bone-marrow-derived PTX3 remains to be addressed[2,85]. Since P-selectin deficiency in $LdlR^{-/-}$[86],$ApoE^{-/-}$[87], and wild-type[88] mice attenuates the development of atherosclerosis, PTX3 protective function could derive from its ability to bind, via its N-linked glycosidic moiety, P-selectin, thus inhibiting leukocyte rolling on endothelium. Moreover, exogenously and endogenously administered PTX3 released from haematopoietic cells provide a negative feedback loop that prevents excessive P-selectin-dependent recruitment of neutrophils in a model of acute lung injury, pleurisy and mesenteric inflammation[89]. These data suggest that PTX3 produced by activated leukocytes might locally dampen neutrophil recruitment and regulate inflammation.

In addition, both IL-4 and Th1-related cytokine interferon-γ inhibit LPS-induced PTX3 expression[55]. Second, anti-inflammatory molecules have been shown to induce PTX3 expression, including IL-10, which sustains LPS-mediated PTX3 expression in DCs[55], GCs, which strongly induce the expression in ECs and fibroblasts[59], and HDL, a class of molecules with anti-atherogenic properties, which induce PTX3 expression *in vitro* in human ECs and *in vivo* in C57BL/6J mouse aortas[83]. Furthermore, several

studies have clearly shown that efficient apoptotic cell clearance inhibits the inflammatory response and reduces the development of atherosclerosis and the size of the necrotic core. PTX3 binds apoptotic cells and regulates their clearance by antigen-presenting DCs[90]. Also, through opsonization of apoptotic cells, PTX3 enhances C1q binding and complement-mediated clearance of the apoptotic debris[21]. Therefore, PTX3 may contribute to limiting the size and complexity of atherosclerotic lesions through both complement-dependent and independent mechanisms. All these data support the cardio- and athero-protective function of PTX3.

In humans, PTX3 plasma levels predict 3-month mortality in patients with AMI[91] and increased tissue and plasma levels of PTX3 were also found in patients with non-rheumatic aortic valve stenosis[92], suggesting a role as diagnostic and prognostic biomarker.

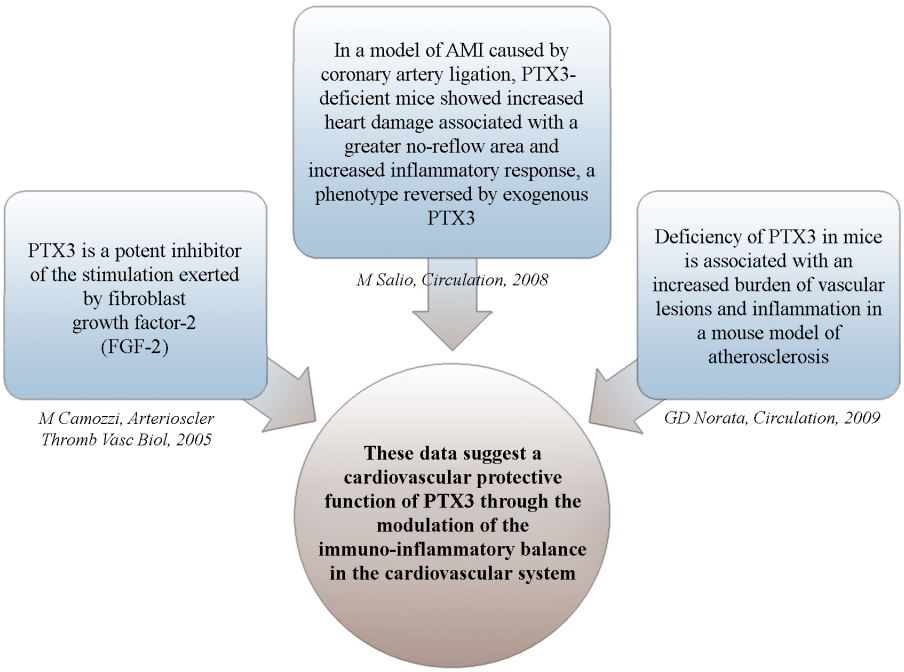

Figure 1.9 | PTX3 is endowed with a potential cardiovascular protective effect.

1.2.6 PTX3 function in tissue remodelling and female fertility

FGF2 is a major heparin-binding angiogenic inducer that promotes cell proliferation, chemotaxis and protease production in cultured ECs and neovascularization *in vivo* during wound healing, inflammation, tumour growth and atherosclerosis. PTX3 recognizes FGF2 with high affinity and specificity, where both proteins are produced by elements of the vessel wall (i.e. ECs and SMCs) during inflammation[71,93]. It is known that FGF2 exerts its activity by interacting with

tyrosine-kinase FGF receptors (FGFRs) and heparan sulfate proteoglycans (HSPGs) on the surface of ECs and SMCs, leading to formation of the proangiogenic HSPG/FGF2/FGFR ternary complex[51]. This process is inhibited by PTX3 in an experimental model of cell – cell adhesion[71]. The molecular mechanisms underneath the inhibitory activity of PTX3 are still unclear; however, recent observations indicate that PTX3 might function by blocking the FGF2–FGFR1 interaction rather than preventing the growth factor association with HSPGs. As a consequence of the HSPG/FGF2/FGFR complex inhibition, PTX3 suppresses the mitogenic activity exerted *in vitro* by FGF2 on cultured bovine, murine and human ECs. Consistent with this, PTX3 inhibits the FGF2-dependent angiogenesis in a number of *in vivo* animal models[94]. Therefore, PTX3 produced by ECs, SMCs and inflammatory cells may affect the autocrine and paracrine activity of FGF2 on endothelium and provide a mechanism for tuning the neo-vascularization process. Actually, the angiogenesis process (such as the generation of new blood vessels from the endothelium of existing vasculature) that occurs under normal physiological conditions in growth and development and is required for successful wound healing, is regulated by the balance between pro- and anti-angiogenic factors.

A putative site of interaction with FGF2 has been identified in the region 97–110 of the PTX3 N-terminal domain[51] (corresponding to the segment between the first and second helices in Fig. 1.6).

The PTX3–FGF2 interaction might also have important implications in the process of blood vessel narrowing that frequently occurs after percutaneous transluminal coronary angioplasty of atherosclerotic arteries. FGF2 is a key factor in restenosis, where it has been shown to promote survival, proliferation and migration of SMCs that express FGFRs[51]. PTX3 prevents the binding of FGF2 to FGFRs in human coronary artery SMCs, thus inhibiting the endogenous FGF2-dependent SMCs proliferation and suppressing the mitogenic and chemotactic activities exerted by exogenous FGF2 on these cells[93]. These observations suggest a novel therapeutic role of PTX3 in the treatment of restenosis after angioplasty[2].

An increasing body of evidence points to PTX3 as an important component of the ECM with novel functional implications in tissue remodelling. Indeed, PTX3 is produced by mouse cumulus cells during cumulus expansion and has been shown to localize within the matrix, playing a non-redundant role in *in vivo* fertilization. In this regard, $Ptx3^{-/-}$ mice generated by homologous recombination display a severe

deficiency in female fertility. This subfertility has been attributed to defective assembly of the viscoelastic HA-rich matrix that forms around the oocyte in the pre-ovulatory follicle (namely, the cumulus oophorus complex), where correct deposition of cumulus matrix is necessary for fertilization *in vivo*. The major component of the cumulus matrix is HA, which is produced by cumulus and granulosa cells[72]. Besides PTX3, other molecules are required for effective incorporation of the newly synthesized HA into the matrix, including the HA-binding protein TSG-6 and the serum proteoglycan IαI. In fact, genetic or functional deletion of IαI or TSG-6 leads to impaired cumulus matrix formation and severe subfertility, a similar phenotype to that observed in $Ptx3^{-/-}$ mice[95]. Current literature supports the hypothesis that heavy chains (HCs) from IαI become covalently attached to HA (i.e. to form HC•HA complexes) through reactions involving TSG-6, which acts as both a cofactor and catalyst. The resulting HC•HA complexes are believed to be crosslinked by PTX3, where the long pentraxin can establish multiple contacts to HCs, thus providing structural integrity to the cumulus matrix. Implicit in this model is the concept that PTX3 has a nodal activity that is dependent on its multimeric organization. Consistent with this, the protein tetramer is the functional molecular unit required for matrix organization. Accordingly, recombinant preparations of the PTX3 N-terminal domain (i.e. that forms tetramers and contain the HC-binding site) have been proved to sustain assembly of the cumulus matrix[73,96].

1.2.7 PTX3 levels in immune and inflammatory disorders and CVD

CRP has been extensively used clinically for over 75 years as a non-specific systemic marker of infection, inflammation and tissue damage. The structural and functional relationship to CRP, now experiencing a renaissance in cardiovascular disorders, and the data obtained in gene-modified mice have prompted studies aimed to assess the usefulness of PTX3 in diverse human pathological conditions. The hypothesis driving much of previous and ongoing efforts is that PTX3, unlike CRP (i.e. made in the liver and induced primarily by IL- 6), may represent a rapid marker for primary local activation of innate immunity and inflammation. Indeed, in all clinical studies conducted so far, the correlation between levels of PTX3 and CRP was loose or non-significant. A second general characteristic emerging from studies of blood levels in murine and human pathology is the rapidity of PTX3 increase, compared to CRP, consistent with its original identification as an immediate early

gene. In man and mouse PTX3 behaves as an acute phase protein because its blood levels, low in normal conditions (about 25 ng/ml in the mouse, <2 ng/ml in humans), increase rapidly (with a maximum at 6–8 h) and dramatically (200–800 ng/ml) during endotoxic shock, sepsis and other inflammatory and infectious conditions. Increased levels of PTX3 have been observed in diverse infectious disorders including sepsis and septic shock, meningococcal disease, tuberculosis (TB) and dengue infection. In some of these cases a significant correlation was found between PTX3 plasma levels and severity of disease[83].

Increased levels of PTX3 have been observed in a restricted set of autoimmune disorders (e.g. in the blood in small vessel vasculitis and psoriasis and in the synovial fluid in rheumatoid arthritis). In small vessel vasculitis, PTX3 levels correlate with clinical activity of the disease and represent a candidate marker for monitoring its progression[90].

Patients with chronic kidney disease (CKD) also show increase in PTX3 plasma levels, with the highest concentrations observed in the group of patients with a more severe disease. Moreover, patients with high PTX3 levels had higher all-cause mortality and cardiovascular mortality, suggesting that PTX3 could have a predictive value of mortality in CKD patients[97]. It has been reported that the concentration of circulating PTX3 increases during pregnancy, a condition that is associated with inflammation. Higher maternal PTX3 levels have been observed in pregnancies complicated by preeclampsia, which represents the clinical manifestation of an endothelial dysfunction that is part of an excessive maternal inflammatory response to pregnancy[98,99]. PTX3 plasma and vaginal levels increase also during pregnancy complicated by spontaneous preterm delivery, in particular in cases of placenta vasculopathy[100]. Furthermore, recent studies have reported the presence of PTX3 in the amniotic fluid and elevated protein concentrations in intra-amniotic inflammation and infection. In contrast, the increased titres of maternal plasma PTX3 observed during labour are not related to either intra-amniotic inflammation or infection[101].

Vessel wall elements (e.g. ECs and SMCs) produce high levels of PTX3 during inflammation. Given the homology to CRP, the prognostic and diagnostic relevance of PTX3 has been investigated in cardiovascular pathology. In this regard, a number of evidences point to PTX3 as a useful marker of vascular integrity, including the observations that high levels of PTX3 are seen in the heart and vascular cells in response to inflammatory signals and ox-LDL and the protein is present in

atherosclerotic lesions[83]. According to this, PTX3 has emerged from several studies as a new candidate prognostic marker in ischaemic heart disorders such as AMI. PTX3 levels increase rapidly in AMI, reaching a peak at around 7 h after the onset of symptoms and do not correlate to those of CRP. Compared with established markers (i.e. CRP, N-terminal pro-brain natriuretic peptide and troponin T), PTX3 has been proposed as the only independent predictor of 3-month mortality in a cohort of 748 patients with AMI and ST elevations[91]. In AMI, immunostaining for PTX3 was observed within and around ischaemic lesions. In early ischaemic lesions, PTX3 was expressed primarily by granulocytes; in more advanced AMI, PTX3 positivity was found in the interstitium and in the cytoplasm of macrophages and the endothelium[102]. Recently, a flow cytometric analysis suggested that in the early phase of AMI (within 6h from the onset of symptoms) circulating neutrophils release preformed PTX3, whereas 48h after the onset of symptoms neutrophil PTX3 returns to normal values[84]. Furthermore, PTX3 is present and localized within and around histological lesions in infectious myocarditis[102].

In a recent prospective study, the correlation of circulating levels of PTX3 to incident CVD in older adults with no clinical symptoms of CVD has been investigated. PTX3 was found to associate with CRP but not with SAP, and with some CVD risk factors (i.e. age, fasting glucose and insulin) and the presence of subclinical CVD. Moreover, PTX3 correlated with CVD death and all-cause death, similarly to CRP. However, unlike CRP, PTX3 associated only with fatal events[103]. Therefore, PTX3 and CRP may reflect different aspects of inflammation in atherosclerosis and perhaps other vascular diseases.

Also heart failure (HF) is characterised by higher than normal circulating concentrations of inflammatory cytokines and CRP. PTX3 levels are higher in patients with HF than in healthy controls, increase with severity of HF and, more interestingly, are independent predictors of the disease outcome. These findings have been extended in two studies that reported a better prediction of HF risk when three biomarkers (i.e. brain natriuretic peptide, heart-type fatty acid-binding protein and PTX3) were combined. Moreover, in the GISSI-HF trial PTX3 was measured at baseline in 1233 patients with chronic HF and found elevated in patients with more severe disease, in particular, in those with advanced age, ventricular dysfunction, worse symptoms and co-morbidities such as atrial fibrillation or diabetes[91].

Circulating PTX3 levels are significantly elevated in patients who undergo percutaneous coronary intervention and in those with unstable angina, thus suggesting that PTX3 may be a candidate marker to predict occurrence of unstable angina[104]. PTX3 has also been proposed as a useful marker for evaluation of inflammatory reaction and neointimal thickening after vascular injury[105].

In conclusion, it is likely that PTX3 act as a molecule at the crossroad between pro-inflammatory and anti-inflammatory stimuli, perhaps by balancing the over-activation of a pro-inflammatory, pro-atherogenic cascade. PTX3 may represent a useful marker of cardiovascular pathology complementary to CRP: being directly produced by damaged tissues, its increase precedes CRP and rapidly reflects the vascular involvement by inflammatory process. In particular, the combination of PTX3 and classical biomarkers showed an incremental diagnostic and prognostic value in several conditions, including sepsis, acute coronary syndromes and chronic heart failure. These data suggest that PTX3 is a novel promising biomarker to provide useful prognostic information for clinical outcomes in these pathologic conditions[106].

1.2.8 *PTX3* genetic variations in innate immunity and fertility

Despite its strong evolutionary conservation, recent studies have reported a certain number of single-nucleotide polymorphisms (SNPs) in the *PTX3* gene, summarized in Table 1.3.

In line with the structural and functional conservation of the gene, most of these genetic variations were found in the non-coding regions and only one of the two described exon substitutions result in amino acid variation of the protein.

Interestingly, some of these polymorphisms, especially when associated in specific haplotypes, have been related to clinical conditions including infections and fertility.

Table 1.3 | *PTX3* single nucleotide polymorphism information.

SNP ID (rs)	Position (bp)	Localization	Alleles	Peptide shift
rs35948036	157154768	Exon 1	T/C	p.Leu16=
rs2305619 *	**157154861**	**Intron 1**	**G/A**	
rs3816527 *	**157155314**	**Exon 2**	**A/C**	**p.Asp48Ala**
rs1840680 *	**157156029**	**Intron 2**	**G/A**	
rs3845978	157159694	Intron 2	C/T	
rs2614	157160999	3'UTR	C/T	
rs6788044	157167340	About 6 kb downstream the 3'UTR (intron 4 of *VEPH1* gene)	T/C	

*The GAG haplotype (rs2305619, rs3816527, rs1840680 SNPs) is protective in *M.tuberculosis* and *P.aeruginosa* infections and it is associated with increased female fertility and LPS-induced PTX3 production.

In 2007, Olesen *et al.* investigated the role of *PTX3* genetic variations in affecting host vulnerability to pulmonary tuberculosis (TB) in West Africans from Guinea-Bissau[107]. This article has been the first work on *PTX3* polymorphisms and the authors demonstrated a difference in *PTX3* haplotype frequencies between cases (N=321) and controls (N=347), revealing a protective effect of a specific haplotype against TB. In particular, they studied 5 *PTX3* SNPs, spanning the 6.7 kb length of the gene: rs2305619, rs3816527, rs1840680, rs3845978, rs2614. Among these SNPs, rs2305619 and rs1840680 showed significant difference between TB cases and controls for both allelic and genotypic distributions using an uncorrected p-value of 0.05. However, none remained significant after Bonferroni's correction. The GAG haplotype, corresponding to rs2305619, rs3816527 and rs1840680 SNPs, showed a significant higher frequency in the control population compared to cases (46.2% vs 36.5%; p=0.0281; OR=0.782), demonstrating a protective effect relative to other haplotypes. The authors did also an LD analysis within *PTX3* gene using the HaploView software. The LD structure in *PTX3* appeared different between cases and controls, with controls having a smaller block size, suggesting either a more recent origin of the case haplotype or a selection maintaining a larger block structure. Data indicating that *PTX3* SNPs affect the susceptibility to pulmonary TB and PTX3 levels correlate with disease activity in West African subjects[108] suggest the relevance of this molecule in innate resistance to TB.

In 2010, Chiarini and colleagues studied the role of *PTX3* polymorphisms in the development of chronic *Pseudomonas aeruginosa* (PA) airway colonization in patients with cystic fibrosis (CF)[109]. The analysis has been conducted in 127 CF Caucasian patients homozygous for F508del (age ≥ 11 years) and on five SNPs in *PTX3* gene, three intronic (rs2305619, rs1840680 and rs3845978) and two exonic (rs35948036 and rs3816527). Statistical analysis has shown that the intronic SNPs rs2305619 and rs1840680 and the exonic rs3816527 are statistically associated (χ^2-test: p=0.012, p=0.016, p=0.038 respectively) with PA colonization. However, after permutation test, PA colonization was significantly associated only with rs2305619. The authors included in the haplotype analysis the three SNPs associated with PA colonization. The GAG haplotype was present in the 58.7% of non-PA patients and in the 42.7% of PA patients (P=0.012). After permutation test, the GAG haplotype preserved a significant p-value (0.030). The linkage disequilibrium (LD) analysis within *PTX3* gene revealed that LD structure in *PTX3* is not different in patients with or without PA colonization. The clinical outcome of pulmonary disease in CF patients is extremely variable, even among patients with the same CFTR genotype. This variability could arise from some combination of environmental and modifier gene effects. As the association between *PTX3* haplotypes and PA infections in CF patients suggests, PTX3 seems to be involved in preventing lung colonization by this opportunistic pathogen.

Since in the mouse PTX3 plays an important role in the formation of the extracellular matrix of the cumulus oophorus in ovarian follicles, as well as implantation and decidualization[72,110,111], May *et al.* tried to understand whether *PTX3* genetic variants could influence female fertility[112]. To study the natural fertility of women the authors conducted the study on subjects from a rural area of Ghana, where the population lives under adverse environmental conditions and the use of contraceptives has been virtually absent. In this association study, 17 SNPs, from HapMap database, in *PTX3* gene and in its flanking regions have been analysed for their potential relationship with the *ex-vivo* LPS-induced PTX3 production and female fertility. The *ex-vivo* LPS-induced PTX3 production was associated with fertility (p=0.040): women who had more than 11 children had highest PTX3 production. The same observation has been done in postmenopausal women, in which fertility can best be observed because reproduction has terminated. No differences have been found in PTX3 production for women with 3-11 children. Six out of the 17 genotyped SNPs have been found to be

associated with either higher or lower LPS-induced PTX3 production. Among them, the only polymorphism mapped within *PTX3* gene is rs3816527: the variant allele carriers (C) have a higher PTX3 production than noncarriers (p=0.035). The strongest association was observed for the rs6788044 SNP that is located in intron 4 of *VEPH1* gene, 6 kb downstream *PTX3* gene: variant allele carriers (C) had significantly higher PTX3 production after LPS stimulation than noncarriers (p=0.003). Genetic variants associated with higher LPS-induced PTX3 production were also associated with a higher number of offspring and vice versa. The rs6788044 SNP associated significantly with higher PTX3 production as well as the amount of offspring (p=0.043). The previously studied GAG haplotype was associated with an increased PTX3 production (p=0.080) and an increased number of offspring (p=0.011). May *et al.* provided the first evidence that PTX3 plays a role in female fertility in humans.

Results from these association studies suggest that genetic variants in *PTX3* are associated with different susceptibility to *M.tuberculosis* and *P.aeruginosa* infections and to fertility as well as different levels of *ex-vivo* LPS-induced PTX3 production.

2- Genetics of complex diseases

2.1 The role of SNPs in susceptibility to complex common diseases

The major impact of genetics on medicine has been so far the identification of visible chromosomal defects and mutations that interfere with the function of a single gene. Diseases caused by these rare variations in a single genetic locus are termed 'Mendelian' or 'single gene' disorders. They exhibit one of the following characteristic patterns of transmission: autosomal dominant, autosomal recessive, X-linked, mitochondrial. On the other hand, most common disorders with adult onset show familial aggregation that usually exhibits a complex inheritance, which is characterized by the additive effects of variations in one or more genes and/or environmental risk factors. Such conditions include for example atherosclerosis, hypertension, hypercholesterolemia, diabetes, obesity and several cancers (Fig. 2.1)[113].

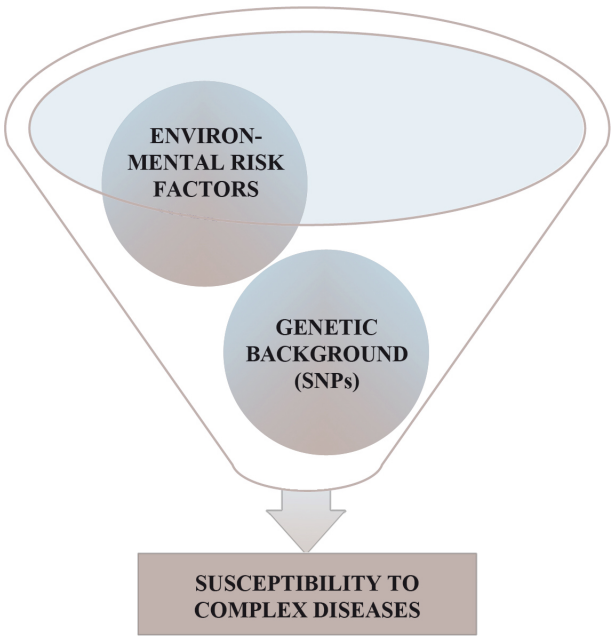

Figure 2.1 | **Multifactorial aetiology of complex diseases.** The susceptibility to complex diseases is given by the combination of genetic and environmental risk factors that, interacting each other, increase the risk of developing such a disease.

Every person in the world, with the exception of identical twins, has a unique genome that shares a 99.9% of identity with the genome of another person. 0.1% of diversity means millions of nucleotide variations among the 3.2 billion base pairs of

the whole human genome. Most of these variations take the form of Single Nucleotide Polymorphisms (SNPs), even if genetic variations include also deletions, insertions and Copy Number Variations (CNV). A SNP is traditionally defined as a variation in DNA sequence that involves only a single nucleotide and that occurs in the population with a minimal frequency of 1% (MAF >1%). These type of genetic variants are expected not only to account for phenotypic variations among individuals, but also for the susceptibility to above-mentioned complex diseases[114].

Probably mutations that cause strongly deleterious phenotypes - as most Mendelian diseases appear to be - are lost because of natural selection. Conversely, variants conferring an advantage are often the basis for evolutionary change and tend to rise rapidly to high frequency, a phenomenon known as genetic hitchhiking[115]. Although the majority of neutral variants, like polymorphisms are often supposed to be, are lost by chance, a minority of them eventually become fixed in the population[116].

The "common disease–common variant" (CD-CV) hypothesis postulates that common polymorphisms (MAF >1%) that have low penetrance might contribute to susceptibility to common diseases[117-119]. Actually, common diseases often have late onset, with modest or no obvious impact on reproductive fitness[120]. Moreover, some alleles that were advantageous or neutral during human evolution might now confer susceptibility to disease because of changes in living conditions accompanying civilization. Finally, disease-associated alleles could be maintained at high frequency if they were under balancing selection, being responsible for a beneficial phenotype (as in sickle-cell disease and malaria resistance). The concept was not that all disease-associated variants at these genes should be common, only that some common variants exist and could be used to pinpoint loci for detailed study[121].

It took a decade to develop the tools and methods required to test the CD-CV hypothesis: (i) catalogues of millions of common variants in the human population, (ii) techniques to genotype these variants in studies with thousands of patients (e.g. Genome-Wide Association Studies, GWAS), and (iii) an analytical framework to distinguish true associations from noise and artefacts[122].

GWAS are proving adept at identifying common variants contributing to the inherited component of common diseases. Almost all such variants seem to have modest effect sizes and, even when combined, their impact on overall population variance and predictive power is limited. There is a marked disparity between the

extent of overall familial aggregation observed for many common diseases and that attributable to variants identified to date. Although the identification of additional common risk variants will explain some of this deficit, one hypothesis anticipates that a significant proportion of this 'missing heritability' will be attributable to low-frequency variants with intermediate penetrance effects, which have been largely refractory to conventional gene-discovery approaches (Fig. 2.2)[123]. Whereas rare variants are more likely to be restricted to specific ethnic groups[124,125], common variants (perhaps 90% of heterozygous sites in each individual) are typically shared among continental populations[122].

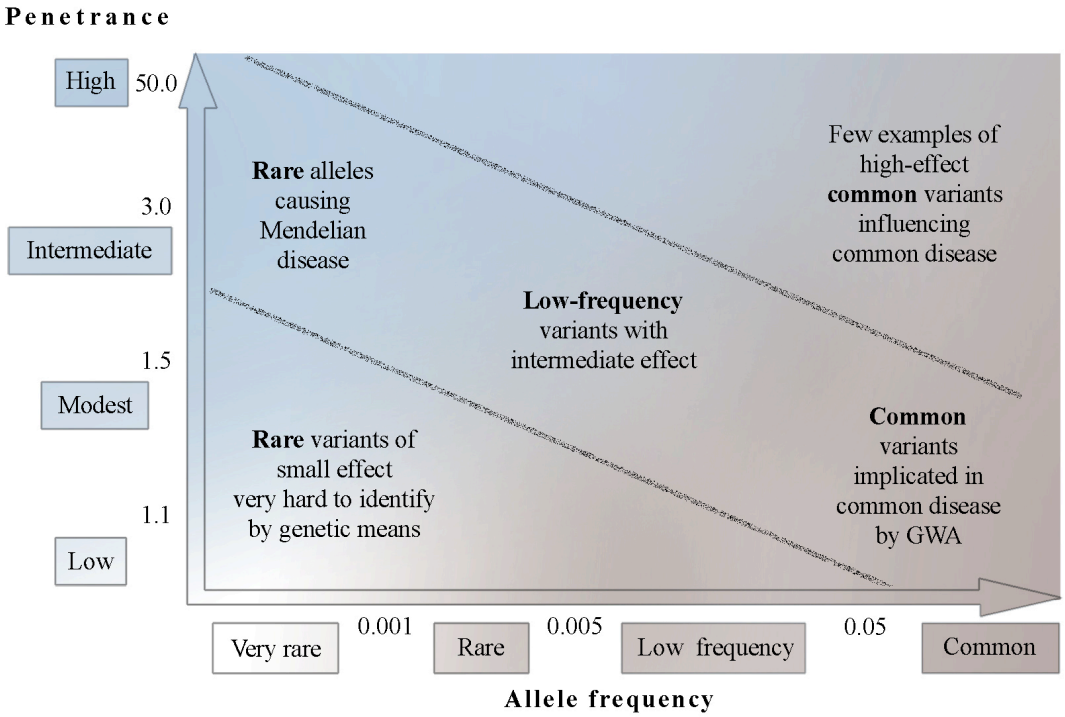

Figure 2.2 | Low-frequency variants and disease susceptibility. Adapted from McCarthy *et al.*[123].

It is important to observe that, usually, each disease-associated variant bears a specific set of common alleles in *cis* at nearby loci (tag SNPs). When considered together, all these SNPs form the so-called "haplotype". This means that genotypes of common SNPs could be inferred from knowledge of only a few empirically determined tag SNPs[126-128]. Because the recombination rate is low (~1 crossover per 100 Mb per generation), disease alleles in the population typically show association with nearby marker alleles for many generations, a phenomenon termed linkage

disequilibrium (LD) (Fig. 2.3)[122]. Actually, when an association between disease susceptibility and a specific SNP is observed, the LD with nearby SNPs has to be considered.

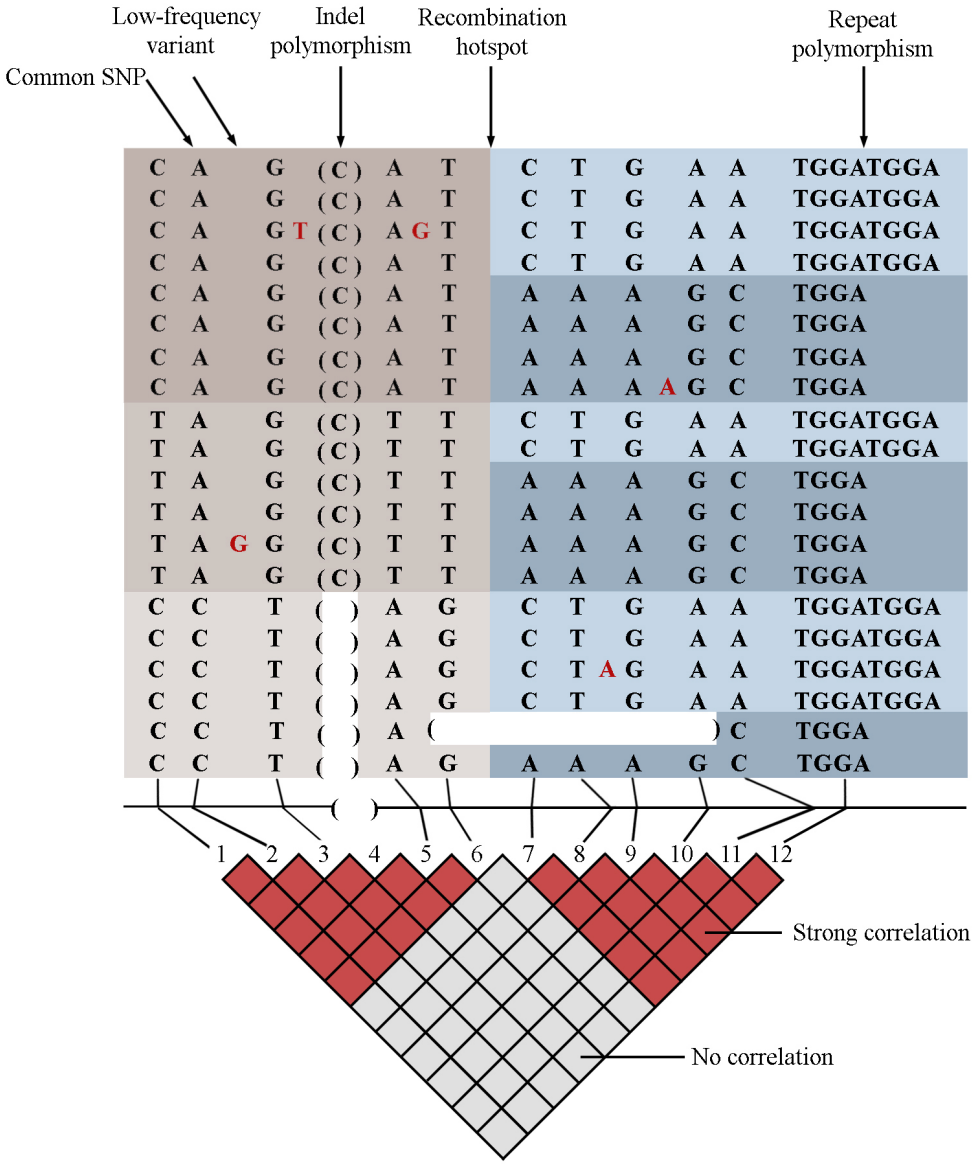

Figure 2.3 | DNA sequence variation in the human genome. Common and rare genetic variations in 10 individuals, carrying 20 distinct copies of the human genome. The amount of variations shown here is typical for a 5-kb stretch of genome and is centered on a strong recombination hotspot. The 12 common variations include 10 SNPs, an insertion-deletion polymorphism (indel), and a tetranucleotide repeat polymorphism. The six common polymorphisms on the left side are strongly correlated. Although these six polymorphisms could theoretically occur in 26 possible patterns, only three patterns are observed (indicated by three different brown shades). These patterns are called haplotypes. Similarly, the six common polymorphisms on the right side are strongly correlated and reside on only two haplotypes (indicated by two different blue shades). The haplotypes occur because there has not been much genetic recombination between the sites. By contrast, there is little correlation between the two groups of polymorphisms, because a hotspot of genetic recombination lies between them. The pairwise correlation between the common sites is shown by the red and white boxes below, with red indicating strong correlation and white indicating weak correlation. In addition to the common

polymorphisms, lower-frequency polymorphisms also occur in the human genome. Five rare SNPs are shown, with the variant nucleotide marked in red and the reference nucleotide not shown. In addition, on the second to last chromosome, a larger deletion variant is observed that removes several kilobases of DNA. Such larger deletion or duplication events (i.e., CNVs) may be common and segregate as other DNA variants. Adapted from Altshuler *et al.*[122].

The International HapMap Project was launched in 2002, with the goal of characterizing SNP frequencies and local LD patterns across the human genome in 270 samples from Europe, Asia, and West Africa. The project genotyped ~1 million SNPs by 2005[129] and more than 3 million by 2007[130].

SNPs, that are located within coding or regulatory regions of a gene, may directly influence phenotype. In fact, when the variation is found in the coding sequence of a gene, it may alter the primary structure and the function of the encoded protein and this could result in a disease manifestation, sometimes with high penetrance. On the other hand, SNPs within regulatory regions tend to have more quantitative effects, for example by altering the expression level of the protein. However, the majority of SNPs are situated in noncoding regions of the genome (Fig. 2.4)[113].

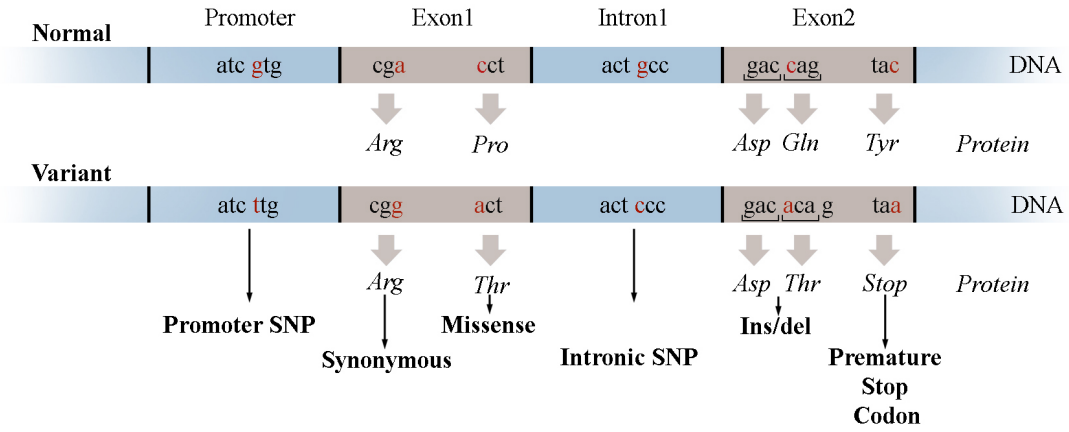

Figure 2.4 | Schematic illustration of SNP types. A SNP in the promoter region of a gene can alter transcription by modifying the binding of specific transcription factors to their binding sites. Within the coding sequence of the gene synonymous SNPs are variations that do not alter the primary structure of the protein, whereas missense SNPs alter a codon, resulting in an amino acid change. The insertion or deletion of a nucleotide in the DNA sequence shifts the reading frame (frameshift) and disrupts the transcription of the remaining sequence. Sometimes a SNP in the coding sequence of a gene can result in the generation of a premature stop codon. Adapted from Ho *et al.*[113].

Noncoding mutations with roles in disease susceptibility will likely open new doors to understanding genome biology and gene regulation. Regulatory variation also suggests different therapeutic strategies: modulating levels of gene expression

may prove more tractable than replacing a fully defective protein or turning off a gain-of-function allele[122].

The association of genetic variants to disease susceptibility provides, on one hand, the opportunity to predict the risk of disease and, on the other hand, novel insights about the mechanism of the disease. The more disease pathways are known, the more prediction, prevention, diagnosis, prognosis and treatment can be improved (Fig. 2.5).

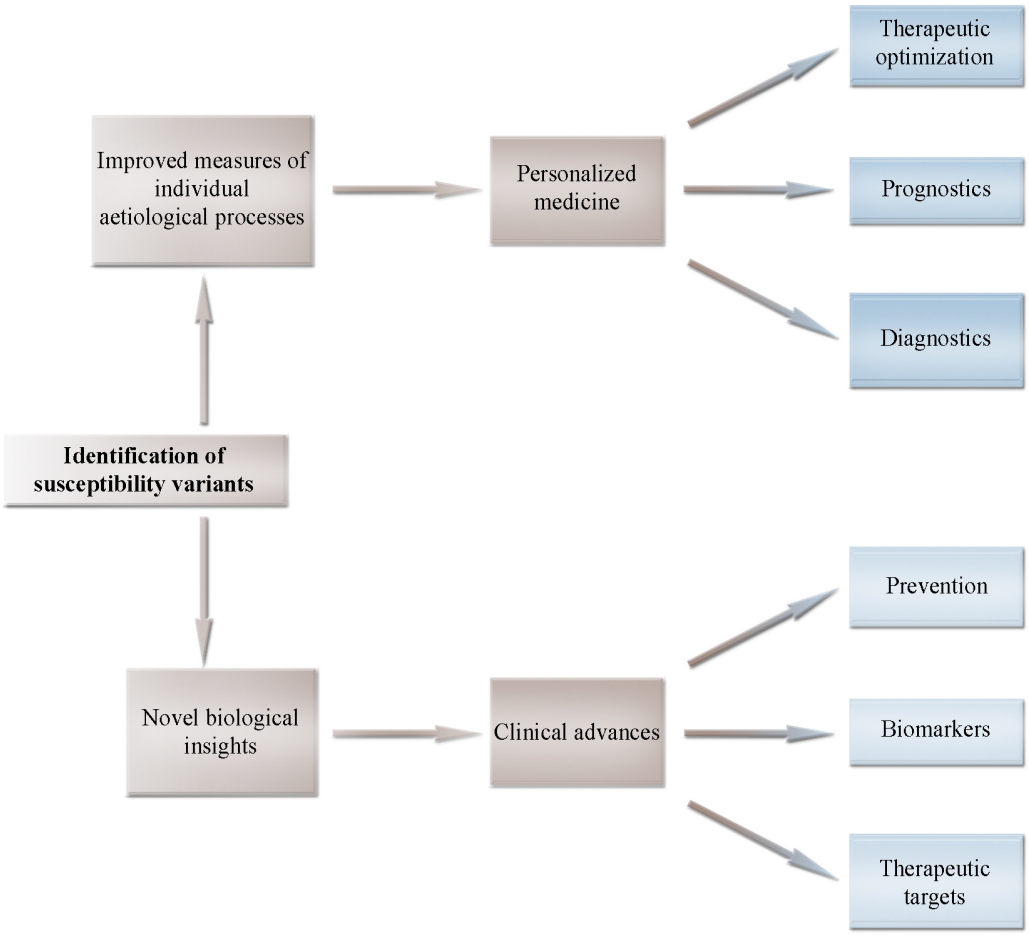

Figure 2.5 | Clinical translation of identified new susceptibility variants. Adapted from McCarthy *et al.*[123].

2.2 Coronary artery disease as a complex trait

Cardiovascular disease (CVD) is the leading cause of mortality in many countries, accounting for 16.7 million deaths each year[131,132]. Coronary artery disease

(CAD) and cerebrovascular disease are the most common forms of cardiovascular disease, and they have severe consequences both for the individual person and society at large. Their underlying pathological process is atherosclerosis, a slowly progressing chronic disorder of large and medium-sized arteries[133]. For many years it was believed that atherosclerosis was merely passive accumulation of cholesterol in the vessel wall. Today, the picture is much more complex, with atherosclerosis being thought of as a chronic inflammatory disease[134].

CAD has long been known to 'run in families'. A small proportion of cases can be attributed to rare, highly penetrant, monogenic effects, but most are multifactorial in aetiology, involving numerous environmental and heritable risk factors.

Molecular genetic studies have identified few mutations that cause premature CAD (Table 2.1). The same studies revealed the heritable basis of several atherosclerosis risk factors (e.g. LDL and HDL cholesterol), which are presumed to explain a fraction of an individual's inherited risk for common CAD. However, most genes involved in susceptibility to multifactorial CAD are unknown[134]. In fact, many studies demonstrated that a family history of CAD/AMI is an independent predictor of CAD risk, even after adjustment for several classical risk factors (sex, age, smoking, LDL and HDL cholesterol and blood pressure)[135,136].

Table 2.1 | **Mendelian diseases that involve premature coronary artery disease.** Adapted from Watkins *et al.*[138].

Disease	Genes	Effect of mutations	OMIM number
Familial hypercholesterolaemia	*LDLR*	Defective binding of LDL by receptor	#143890
Familial defective APOB	*APOB*	Reduced binding affinity of APOB to LDLR	#144010
Autosomal recessive hypercholesterolaemia	*ARH*	Defective endocytosis of LDLR	#603813
APOA1 deficiency	*APOA1*	Deletion or loss-of- function mutation that leads to very low HDL	#107680
Tangier disease	*ABCA1*	Impaired cholesterol efflux in macrophages (foam cells)	#205400
Homocystinuria	*CBS*	Homocysteine increases thrombotic tendency	#23620

Although a family history of CAD is considered a classical risk factor, it is complex to associate the individual genetic background to the risk of developing atherosclerosis and subsequently Acute Myocardial Infarction (AMI)[113]. We are probably far from the possibility to quantify the risk to develop atherosclerosis or

AMI based on the genetic fingerprint, but this type of information could be useful for stratifying the population and perform a primary prevention and for improving our understanding of its pathogenesis.

Notably, the genetic architecture of CAD susceptibility is extremely complex. First, the list of known risk factors is long and many of these traits have their own complex genetic basis. Second, CAD is rare before the age of 50 years, and is therefore confined to aged populations. Consequently, it can be considered an entirely modern disease not directly subjected to selective pressure as it is unlikely to have an effect on reproductive success. Variants that confer either susceptibility or protection to CAD might therefore have evolved neutrally in the past[137]. It is possible that CAD-associated variants have pleiotropic effects, thus affecting the susceptibility to more 'ancient' diseases (for example, infectious disease)[138].

The strategies used to study the genetic factors of CAD have evolved rapidly in recent years due to advances in high-throughput genomic technologies, sharing of data in large scale consortia[129], and statistical genomics. Two different approaches can be used for investigating the genetic basis of CAD: linkage analysis and association studies. The genes identified through these combined approaches have diverse functions: they include transcription factors that are implicated in vasculogenesis, signalling molecules that are involved in inflammation, innate and adaptive immunity, and novel apolipoproteins, as well as genes for which the physiological roles are as yet unclear[138].

2.2.1 Linkage analysis

A linkage analysis is based on the assumption that a genetic linkage exists between disease-associated variations and common polymorphic variants, named "markers". These variations are shared by affected individuals in a large pedigree (in which the disease of interest is prevalent) more often than predicted by Mendelian segregation or chance. The probability that the marker and a CAD susceptibility locus are physically linked on the genetic map (and are likely inherited together) is estimated through the LOD score (the base 10 logarithm of the odds score)[113,139].

The linkage study approach is classically successful in the identification of causal variants for conditions with strong heritability, particularly monogenic Mendelian disorders[139]. This study has a very low statistical power to detect genetic variations

with low penetrance and small effect on susceptibility for complex diseases. Hence, linkage studies have produced very few reproducible results for CAD[140].

2.2.2 Association studies

Association studies compare the allelic and genotype frequencies of genetic variants among affected and unaffected individuals[122]. Association studies have more statistical power than linkage analysis in detecting genetic contributions to complex traits[141]. The large sample size used in the association studies increase the likelihood of identifying common genetic variants with small effects on CAD susceptibility[113].

Association studies can be conducted on genetic variations in a specific gene, which is likely involved in the pathogenesis of the considered phenotype (candidate-gene association study), or on common genetic variants across the whole genome (GWAS).

The major advantage of candidate-gene studies is their avoidance of the multiple-testing problem that complicates GWAS. However, very few associations identified by candidate-gene studies have been consistently replicated for CAD[142]. Actually, the reproducibility of candidate-gene association studies is affected by many factors, such as different patterns of SNP in different populations, excessive false-positive rate, confounding genetic/environmental factors, and the phenotypic heterogeneity of CAD. Meta-analyses have been performed to improve the statistical significance of candidate-gene association studies, but are limited by differences in study designs and phenotype definitions across studies[113].

ApoE genetic variants have been successfully correlated to CAD susceptibility through the candidate-gene association study approach[143].

Limitations of candidate-gene association studies and advances in bioinformatics and high-throughput genomic technology have facilitated the transition from candidate-gene strategies to GWAS to search for susceptibility genes for common CAD.

The GWA approach is similar to that of candidate-gene association studies in that it examines large numbers of unrelated individuals. However, this approach involves the genotyping of an unbiased set of hundreds of thousands of markers spanning across the whole genome. As the genome-wide approach does not depend on any prior assumptions, it has a much greater ability to detect novel susceptibility variants that may lead to an improved understanding of disease pathobiology. In addition,

large-scale GWAS have greater power than linkage studies to identify genetic variants with small effects on CAD susceptibility[119,138]. Large sample sizes are required to detect genes with statistically significant associations that contribute with a small effect[144].

To date, the strongest and most consistent association between a genetic locus and CAD identified by the GWA approach is on chromosome 9 (chr9p21), reported in 2007[145-148]. The variant within the chromosome 9p21 locus that confers CAD risk is extremely common, with 75% of the Caucasian population carrying at least 1 allele[149]. The AMI risk conferred by this 9p21 variant is independent of established cardiovascular risk factors, but its effect is small (each risk allele increases the probability of AMI by 10% to 30% per allele)[150]. The risk may be different for different ethnic groups: actually, the 9p21 locus is a common risk factor for East Asians, but not for African-Americans[145]. Interestingly, there is no evidence that this susceptibility locus influences intermediate phenotypes such as established risk factors for CAD, suggesting that it may contribute to CAD through novel mechanisms[149]. 9p21 is a region without a known protein-encoding gene[150], but contains a large antisense noncoding RNA gene (ANRIL) that affects the regulation of other genes[151].

2.3 Inflammation in atherosclerosis

Research has shown that inflammation plays a key role in CAD and other manifestations of atherosclerosis. Immune cells dominate early atherosclerotic lesions, their effector molecules accelerate progression of the lesions and activation of inflammation can elicit acute coronary syndromes. Atherosclerosis, a slowly progressing chronic disorder of large and medium-sized arteries, is an inflammatory disease in which immune mechanisms interact with risk factors to initiate, propagate, and activate lesions in the arterial tree[133,138].

Our views of the pathophysiology of atherosclerosis have evolved substantively over the past century. The link between lipids and atherosclerosis dominated our thinking until the 1970s, based on strong experimental and clinical relationships between hypercholesterolaemia and atheroma[152]. The emerging knowledge of vascular biology led to a focus on growth factors and proliferation of smooth muscle cells in the 1970s and 1980s[153]. Hence, for many years it was believed that atherosclerosis was merely passive accumulation of cholesterol in the vessel wall.

Today, the picture is much more complex, with atherosclerosis being thought of as a chronic inflammatory disease involving innate and adaptive immune mechanisms[134].

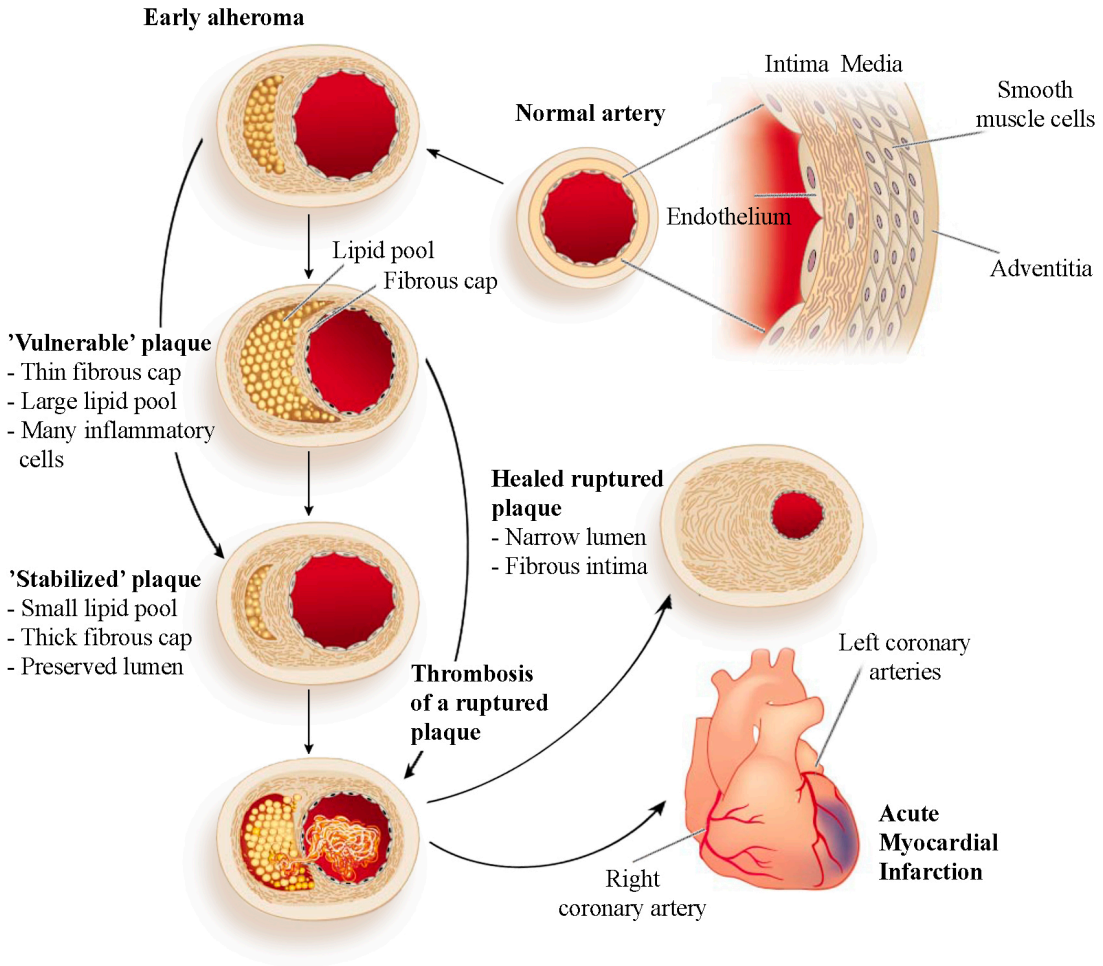

Figure 2.6 | Schematic of the life history of an atheroma. The normal human coronary artery has a typical trilaminar structure. The endothelial cells in contact with the blood in the arterial lumen rest upon a basement membrane. The intimal layer in adult humans generally contains a smattering of smooth muscle cells scattered within the intimal extracellular matrix. The internal elastic lamina forms the barrier between the tunica intima and the underlying tunica media. The media consists of multiple layers of smooth muscle cells, much more tightly packed than in the diffusely thickened intima, and embedded in a matrix rich in elastin as well as collagen. In early atherogenesis, recruitment of inflammatory cells and the accumulation of lipids leads to formation of a lipid-rich core, as the artery enlarges in an outward, ablumenal direction to accommodate the expansion of the intima. If inflammatory conditions prevail and risk factors such as dyslipidaemia persist, the lipid core can grow, and proteinases secreted by the activated leukocytes can degrade the extracellular matrix, while pro-inflammatory cytokines such as interferon-γ (IFN-γ) can limit the synthesis of new collagen. These changes can thin the fibrous cap and render it friable and susceptible to rupture. When the plaque ruptures, blood coming in contact with the tissue factor in the plaque coagulates. Platelets activated by thrombin generated from the coagulation cascade and by contact with the intimal compartment instigate thrombus formation. If the thrombus occludes the vessel persistently, an acute myocardial

infarction can result (the dusky blue area in the anterior wall of the left ventricle, lower right). The thrombus may eventually resorb as a result of endogenous or therapeutic thrombolysis. However, a wound healing response triggered by thrombin generated during blood coagulation can stimulate smooth muscle proliferation. Platelet-derived growth factor (PDGF) released from activated platelets stimulates smooth muscle cell migration. Transforming growth factor-β (TFG-β), also released from activated platelets, stimulates interstitial collagen production. This increased migration, proliferation and extracellular matrix synthesis by smooth muscle cells thickens the fibrous cap and causes further expansion of the intima, often now in an inward direction, yielding constriction of the lumen. Stenotic lesions produced by the lumenal encroachment of the fibrosed plaque may restrict flow, particularly under situations of increased cardiac demand, leading to ischaemia, commonly provoking symptoms such as angina pectoris. Advanced stenotic plaques, being more fibrous, may prove less susceptible to rupture and renewed thrombosis. Lipid lowering can reduce lipid content and calm the intimal inflammatory response, yielding a more 'stable' plaque with a thick fibrous cap and a preserved lumen. Adapted from Libby *et al.*[153].

After the loss of the normal barrier function of the endothelium, the atherosclerotic plaque is characterized by an accumulation of lipids in the artery wall, together with infiltration of leukocytes, such as macrophages, T cells and mast cells, and the formation by vascular smooth muscle cells of a fibrous cap composed mostly of collagen. Early lesions called 'fatty streaks' consist of subendothelial depositions of lipids, macrophage foam cells loaded with cholesterol and T cells. Over time, a more complex lesion develops, with apoptotic as well as necrotic cells, cell debris and cholesterol crystals forming a necrotic core in the lesion. This structure is covered by a fibrous cap of variable thickness and its 'shoulder' regions are infiltrated by activated T cells, macrophages and mast cells, which produce proinflammatory mediators and enzymes[154].

Plaques can remain silent or growth causing stenosis (narrowing of the lumen), hence contributing to ischemia in the surrounding tissue and to angina. Thrombosis is triggered at the surface of the plaque. Myocardial infarction occurs when the atheromatous process prevents blood flow through the coronary artery. It was previously thought that progressive luminal narrowing from continued growth of smooth muscle cells in the plaque was the main cause of infarction. Angiographic studies have, however, identified culprit lesions that do not cause marked stenosis,[155] and it is now evident that the activation of plaque, rather than stenosis, precipitates ischemia and infarction[156] (Fig. 2.6).

There are two major causes of coronary thrombosis: plaque rupture and endothelial erosion. Plaque rupture, which is detectable in 60 to 70% of cases[157], is dangerous because it exposes prothrombotic material from the core of the plaque - phospholipids, tissue factor, and platelet-adhesive matrix molecules - to the blood.

Ruptures preferentially occur where the fibrous cap is thin and partly destroyed. At these sites, activated immune cells are abundant[158]. They produce numerous inflammatory molecules and proteolytic enzymes that can weaken the cap and activate cells in the core, transforming the stable plaque into a vulnerable, unstable structure that can rupture, induce a thrombus that may either obliterate the lumen immediately or detach to become an embolus that can block blood flow distal to its point of origin[134].

Superficial erosion, or microscopic areas of desquamation of endothelial cells that form the monolayer covering the intima, occurs frequently in both humans and animals with experimentally induced atherosclerosis. Such areas of limited endothelial desquamation often form the nidus of a platelet thrombus as they uncover subendothelial collagen and von Willebrand factor that promote platelet adhesion and activation[159]. Although common and most often asymptomatic, such superficial erosion may account for approximately 25% of fatal coronary thrombosis[153].

Commonly used experimental mouse models, such as mice rendered hypercholesterolemic by targeted deletion of genes encoding molecules involved in cholesterol metabolism (such as apolipoprotein E, ApoE$^{-/-}$ mice) or the receptor for low-density lipoprotein (LDL; Ldlr$^{-/-}$ mice), are very useful for delineating the mechanisms of disease initiation and early growth. However, they are not particularly helpful in studies of plaque rupture and thrombosis, which are still based mainly on histopathological and clinical studies. The field clearly needs reliable, quantitative models for this phase of the disease[134].

2.3.1 Inflammation in initiation of the atherosclerotic process

Studies in animals and humans have shown that hypercholesterolemia causes focal activation of endothelium in large and medium-sized arteries. The infiltration and retention of LDL in the arterial intima initiate an inflammatory response in the artery wall[160,161]. Modification of LDL, through oxidation or enzymatic attack in the intima, leads to the release of phospholipids that can activate endothelial cells[161], preferentially at sites of hemodynamic strain[162]. Patterns of hemodynamic flow typical for atherosclerosis-prone segments (low average shear but high oscillatory shear stress) cause increased expression of adhesion molecules and inflammatory genes by endothelial cells[163]. Therefore, hemodynamic strain and accumulation of lipids may initiate an inflammatory process in the artery.

The platelet is the first blood cell to arrive at the scene of endothelial activation. Its glycoproteins Ib and IIb/IIIa engage surface molecules on the endothelial cell, which may contribute to endothelial activation. Inhibition of platelet adhesion reduces leukocyte infiltration and atherosclerosis in hypercholesterolemic mice[164].

Activated endothelial cells express several types of leukocyte adhesion molecules, which cause blood cells rolling along the vascular surface to adhere at the site of activation[165]. Since vascular-cell adhesion molecule 1 (VCAM-1) is typically up-regulated in response to hypercholesterolemia, cells carrying counter-receptors for VCAM-1 (i.e., monocytes and lymphocytes) preferentially adhere to these sites[166].

The mechanism of VCAM-1 induction early after initiating an atherogenic diet probably depends on inflammation instigated by modified lipoprotein particles accumulating in the arterial intima in response to the hyperlipidaemia. Constituents of modified lipoprotein particles can induce transcriptional activation of the *VCAM-1* gene mediated in part by NF-κB[167]. Pro-inflammatory cytokines such as IL-1β or TNFα induce VCAM-1 expression in endothelial cells by this pathway. Human atherosclerotic lesions contain these cytokines. Thus, pro-inflammatory cytokines may link hypercholesterolaemia to VCAM-1 expression[153].

In addition to VCAM-1, P- and E-selectin also seem to contribute to leukocyte recruitment in atherosclerosis-susceptible mice[86,168].

2.3.2 Inflammation in atheroma progression and complication

Morphologic studies have established that, once adherent to the endothelial cell, leukocytes enter the intima by diapedesis between endothelial cells at their junctions. Investigators have defined families of chemoattractant cytokines (chemokines) capable of recruiting leukocytes into the arterial intima. For example, monocyte chemoattractant protein-1 (CCL2/MCP-1), overexpressed in human and experimental atheroma, can recruit the mononuclear phagocytes that characteristically accumulate in the nascent atheroma. Recent work using compound mutant mice lacking MCP-1 or its receptor CCR2, and susceptible to atherosclerosis (ApoE$^{-/-}$ or Ldlr$^{-/-}$ mice), has shown striking decreases in mononuclear phagocyte accumulation and local lipid levels[169,170]. IL-8 may have a similar role in leukocyte chemotaxis during atherogenesis[171].

Once resident in the arterial intima, monocytes acquire the morphological characteristics of macrophages, undergoing a series of changes that lead ultimately to

foam cell formation. Monocytes increase expression of scavenger receptors from modified lipoproteins such as the scavenger receptor A (SRA) and CD36, and then internalize modified lipoproteins, such that cholesteryl esters accumulate in cytoplasmic droplets. These lipid-laden macrophages, known as foam cells, characterize the early atherosclerotic lesion. Macrophages within atheroma also secrete a number of growth factors and cytokines involved in lesion progression and complication (see below). In addition, macrophages replicate within the intima.

Years ago macrophage colony-stimulating factor (M-CSF) has been identified as a candidate activator of several of the steps that stimulate transition of the monocyte to the lipid-laden macrophage. M-CSF augments SRA expression, increases production of cytokines and growth factors by these cells, and also serves as a survival and comitogenic stimulus. Both experimental and human atherosclerotic plaques overexpress M-CSF[172,173].

Granulocyte–macrophage colony-stimulating factor (GM-CSF) may also promote inflammation in the atheroma. GM-CSF aids the survival of a population of mononuclear phagocytes that contain myeloperoxidase, an enzyme that gives rise to the pro-oxidant hypochlorous acid, a potential source of oxidative stress and inflammation in the human plaque[174].

The defence of the normal artery depends on innate immune responses mounted by endothelial cells and, after an inflammatory challenge, by macrophages and other cells of the immune response that are recruited to the artery wall. Such innate immune responses also have a major role in the initiation of atherosclerosis and they involve pattern-recognition receptors (PRRs)[175].

Scavenger receptors that internalize modified LDL particles are multifunctional PRRs that clear the local environment of cell debris, internalize microbes and assist in adhesion and antigen presentation. Scavenger receptors that recognize oxidation-specific epitopes of oxLDL include SRA-1 and SRA-2, MARCO, CD36, SR-B1, LOX-1 and PSOX. Although these receptors undoubtedly serve a major role as mediators of intracellular cholesterol accumulation, their importance in atherosclerosis remains unclear, and gene-knockout studies of hypercholesterolemic mice have provided contradictory results[176].

The endothelium of normal and atherosclerotic arteries expresses a broad repertoire of signalling PRRs, including Toll Like Receptors TLR1, TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9. Monocyte-derived macrophages recruited to forming

lesions also express a broad range of TLRs as well as other signalling PRRs[177]. Knockout studies of hypercholesterolemic mice have demonstrated a major proatherosclerotic role for MyD88, a key adaptor protein in the signalling cascades of most TLRs[178,179]. Targeted deletion of the gene encoding TLR4 also results in less atherosclerosis, albeit to a smaller extent. Of note, MyD88 also participates in the signal-transduction pathway downstream of the receptors for IL-1 and IL-18, two proatherosclerotic cytokines[180,181]. Therefore, part of the diminished disease observed in MyD88-deficient mice probably also reflects the loss of signalling by IL-1β and IL-18[134].

Moreover, oxidized LDL, and components thereof, can ligate particular TLRs (like TLR2 and 4)[182,183]. Further studies will be needed to clarify the role of these ligand-receptor interactions[134].

2.3.3 Adaptive immune system in atherosclerosis

Components of adaptive immunity are present in human lesions throughout the course of atherosclerosis, and several studies have indicated an important role for antigen-specific adaptive immune responses in the atherogenic process. Studies of mouse models of atherosclerosis, such as ApoE$^{-/-}$ or Ldlr$^{-/-}$ mice, in combination with mice deficient in both B cells and T cells, have demonstrated a substantial role for the adaptive arm of immunity in atherosclerosis[184].

Some experiments have suggested that B cells have a protective role: antibodies to oxLDL in particular are atheroprotective. Many experimental studies of rabbits and mice in which oxLDL is used for immunization have shown a positive correlation between high titers of anti-oxLDL and the degree of protection against atherosclerosis[185,186]. As is often the case, the situation is more complex in humans, with various studies showing a positive or negative correlation or no correlation between anti-LDL titers and atherosclerosis or its manifestations[187-189]. Interestingly, titers of IgM and IgG antibodies to oxLDL have been found to show differences in their associations with CAD, which suggests that their biological roles also differ[190].

A T-cell infiltrate is always present in atherosclerotic lesions. Such infiltrates are predominantly CD4$^+$ T cells, which recognize protein antigens presented to them as fragments bound to major-histocompatibility-complex (MHC) class II molecules. CD4$^+$ T cells reactive to the disease-related antigens oxLDL, heat-shock protein 60, and C*hlamydia* proteins have been cloned from human lesions[191-193]. In fact, several

studies have linked infections to atherosclerosis and CAD. In particular, *Chlamydia pneumoniae* and *cytomegalovirus* may contribute to CAD, but it is unlikely that a single microbe causes atherosclerosis. Instead, the total burden of infection at various sites may affect the progression of atherosclerosis and elicit clinical manifestations[194].

A minor T-cell subpopulation, natural killer T cells, is prevalent in early lesions. Natural killer T cells recognize lipid antigens, and their activation increases atherosclerosis in $ApoE^{-/-}$ mice[195]. $CD8^+$ T cells restricted by MHC class I antigens are also present in atherosclerotic lesions. These cells typically recognize viral antigens, which may be present in the lesions. Activation of $CD8^+$ T cells in $ApoE^{-/-}$ mice can cause the death of arterial cells and accelerate atherosclerosis[196].

Atherosclerosis is driven by the T helper type 1 (Th1) response. Interferon-γ, the signature Th1 cytokine, is present in the human plaque and has pathogenic effects, including less collagen fiber formation, higher expression of MHC class II, enhanced protease and chemokine secretion, upregulation of adhesion molecules, induction of proinflammatory cytokines, and enhanced activation of macrophages and endothelial cells[154]. Mice deficient in interferon-γ or its receptor have a lower lesion burden, and mice that receive interferon-γ have larger lesions than those of control mice[197,198]. Injection of IL-12 also promotes the formation of early lesions[199], whereas targeted deletion of the gene encoding IL-12 or vaccination against IL-12 inhibits early but not late lesion development[200,201]. Furthermore, mice lacking IL-18, a Th1-promoting cytokine, have smaller lesions[181], whereas mice treated with IL-18 have more atherosclerosis[202].

Collectively these data demonstrate that Th1 cells have a major role in the pathogenesis of atherosclerosis. IL-4, the signature cytokine of the Th2 lineage, is not frequently observed in human plaques[203], and experimental studies examining the involvement of Th2 cells are contradictory, with some showing proatherosclerotic effects[200,204] and others showing protective effects[205] or no significant effect[206]. IL-33, a powerful inducer of Th2 responses, results in less atherosclerosis in $ApoE^{-/-}$ mice[183]. On balance, then, the role of Th2 immune responses in atherosclerosis remains unclear.

T-cell cytokines cause the production of large amounts of molecules downstream the cytokine cascade. As a result, elevated levels of IL-6 and CRP may be detected in the peripheral circulation. In this way, the activation of a limited number of immune

cells can initiate a potent inflammatory cascade, both in the forming lesion and systemically[133].

2.3.4 Inflammation and plaque disruption

Growth factors elaborated by macrophages in the atherosclerotic intima supposedly stimulated the replication of smooth muscle cells, which accumulate in the plaque and lay down an abundant extracellular matrix[134].

Among the forms of plaque disruption, we best understand fracture of the fibrous cap. Interstitial collagen molecules confer most of the tensile strength on the fibrous cap, and several tightly regulated processes determine the level of collagen crucial for stability of this structure. Certain pro-inflammatory cytokines, such as IFN-γ, can inhibit collagen production by smooth muscle cells, the principle source of this extracellular matrix macromolecule in the arterial wall. Interstitial collagen fibrils usually resist proteolytic degradation, and only a limited number of interstitial collagenases can make an initial proteolytic nick in the collagen chains that make up the triple helical collagen fibril[153].

Two types of proteases have been implicated as key players in plaque activation: matrix metalloproteinases (MMPs) and cysteine proteases[207,208]. Several members of these families of enzymes occur in the plaque and may degrade its matrix. MMP activity is controlled at several levels: inflammatory cytokines induce the expression of *MMP* genes, plasmin activates proforms of these enzymes, and inhibitor proteins (tissue inhibitor of metalloproteinase) suppress their action. Similarly, cysteine proteases are induced by certain cytokines and checked by inhibitors termed "cystatins." Several of these molecules play decisive roles in the formation of aneurysms, as shown by experiments in gene-targeted mice. However, mechanistic studies in models of atherosclerosis have yielded complex results, with certain MMPs reducing rather than increasing the size of the lesions. At the same time, these enzymes clearly affect the composition of plaque. Therefore, they may represent future therapeutic targets. Study of plaque rupture in animal models should be helpful in determining the role of these proteases in the activation of plaque and myocardial infarction[133].

3-microRNAs: new regulators of gene expression

The recent discovery of microRNAs (miRNAs) has revealed a new level of gene expression regulation that affects many biological systems, including the mammalian immune system[209,210]. miRNAs are small, single-stranded non-coding RNAs, many of which have been highly conserved throughout evolution. They function by directly binding to the 3′ untranslated region (3'UTR) of specific target mRNAs, leading to the repression of protein expression. So far, ~1500 different miRNAs have been identified in the human genome. Each miRNA could have the potential to repress the expression of many, perhaps hundreds, of target genes, highlighting the extent of this form of regulation in mammals. More than 100 different miRNAs are expressed by cells of the immune system; they have the potential to broadly influence the molecular pathways that control the development and function of innate and adaptive immune responses. The expression of miRNAs is also markedly deregulated in cancers of immunological origin, in which they are thought to have tumour suppressive or tumour promoting activities depending on the nature of their specific target mRNAs[211].

3.1 The case of *lin-4* and the microRNA discovery

The investigation that led to the discovery of microRNAs was conducted by Victor Ambros, Rosalind Lee and Rhonda Feinbaum. They found that *lin-4*, a gene known to control the timing of *Caenorhabditis elegans* larval development, does not code for a protein but instead produces a pair of small RNAs[212]. One RNA is approximately 22 nucleotides (nt) in length, and the other is approximately 61 nt-long; the longer one was predicted to fold into a stem loop proposed to be the precursor of the shorter one. The authors then noticed that these *lin-4* RNAs had antisense complementarity to multiple sites in the 3'UTR of the *lin-14* gene[212,213]. This complementarity fell in a region of the 3'UTR previously proposed to mediate the repression of *lin-14* by the *lin-4* gene product[214]. The scientists demonstrated the importance of these complementary sites for regulation of *lin-14* by *lin-4*, showing also that this regulation substantially reduces the amount of LIN-14 protein without noticeable change in levels of *lin-14* mRNA. Together, these discoveries supported a model in which the *lin-4* RNAs pair to the *lin-14* 3'UTR to specify translational

repression of the *lin-14* message as part of the regulatory pathway that triggers the transition from cell divisions of the first larval stage to those of the second[212,213].

The shorter *lin-4* RNA is now recognized as the founding member of an abundant class of tiny regulatory RNAs called microRNAs or miRNAs[215-217].

3.2 The microRNA genes and function

In *C. elegans* another gene, *let-7*, was found to encode for a ~22 nt regulatory RNA. The *let-7* RNA acts to promote the transition from late-larval to adult cell fates in the same way that the *lin-4* RNA acts earlier in development to promote the progression from the first larval stage to the second[218,219]. Furthermore, homologs of the *let-7* gene were soon identified in the human and fly genomes, and *let-7* RNA itself was detected in human, *Drosophila*, and eleven other bilateral animals[220].

Because of their common roles in controlling the timing of developmental transitions, the *lin-4* and *let-7* RNAs were dubbed small temporal RNAs (stRNAs), with anticipation that additional regulatory RNAs of this type would be discovered[220]. Indeed, less than one year later, three labs cloning small RNAs from flies, worms, and human cells reported a total of over one hundred additional genes for tiny noncoding RNAs[215-217]. The RNA products of these genes resembled the *lin-4* and *let-7* stRNAs in that they were ~22 nt endogenously expressed RNAs, potentially processed from one arm of a stem loop precursor, and they were generally conserved in evolution. But unlike *lin-4* and *let-7* RNAs, many of the newly identified ~22 nt RNAs were not expressed in distinct stages of development and instead were more likely to be expressed in particular cell types. Thus the term microRNA was used to refer to the stRNAs and all the other tiny RNAs with similar features but unknown functions[215-217]. Actually, different miRNA expression profiles can be detected according to the development stage, the differentiation stage or the cell type. A distinct pattern of miRNA expression provides the opportunity for “micromanaging” the output of the transcriptome[221].

Like *C. elegans lin-4* and *let-7*, most miRNA genes come from regions of the genome quite distant from previously annotated genes, implying that they derive from independent transcription units[215-217]. About a quarter of the human miRNA genes are in the introns of pre-mRNAs. These are preferentially in the same orientation as the predicted mRNAs, suggesting that most of these miRNAs are not transcribed from their own promoters but are instead processed from the introns. This arrangement

provides a convenient mechanism for the coordinated expression of a miRNA and a protein. Regulatory scenarios are easy to imagine in which such coordinate expression could be useful, which would explain the conserved relationships between miRNAs and host mRNAs[221].

miRNA genes can be clustered in the genome with an arrangement and an expression pattern implying transcription as a multi-cistronic primary transcript[215,216] or isolated, like the majority of worm and human miRNA genes are[222,223]. In contrast, in *Drosophila* over half of the known miRNAs are clustered[224]. The miRNAs within a genomic cluster are often, though not always, related to each other; and related miRNAs are sometimes but not always clustered[215,216]. This example illustrates the possibility that even in cases where clustered genes have no apparent sequence homology, they may share functional relationships[221].

3.3 The microRNA biogenesis and mechanism of action

Our knowledge of miRNA biogenesis and mechanisms of target mRNA regulation by miRNAs is expanding in parallel with our understanding of their physiological roles. The processes of miRNA biogenesis and target mRNA repression (Fig. 3.1) have been intensively studied in recent years[225]. miRNAs are encoded by genomic DNA and are most commonly transcribed by RNA polymerase II. miRNAs are processed from their primary transcripts (known as pri-miRNAs) by the microprocessor complex, which consists of the RNase III type endonuclease Drosha (also known as ribonuclease 3) and its partner DiGeorge syndrome critical region gene 8 (DGCR8), and recent data suggest that this occurs co-transcriptionally[226]. The precursor-microRNA (pre-miRNA) generated by the microprocessor complex is a 70 nt stem loop that is then actively exported from the nucleus to the cytoplasm by exportin 5. On reaching the cytoplasm, further processing of the pre-miRNA is carried out by Dicer, another RNase III type endonuclease, and TAR RNA-binding protein (TRBP, also known as TARBP2) resulting in the formation of a miRNA duplex 20 nt-long. The duplex is unwound, and one strand, the guide strand, is packaged into the RNA-induced silencing complex (RISC), which comprises Argonaute and other proteins. The miRNA then guides the RISC to its target 3′ UTRs, leading to inhibition of translation and/or a decrease in mRNA stability as a result of accelerated uncapping and deadenylation[227,228]. The deadenylation process, through which the polyA tail is removed from the mRNA, is reached by fostering the activity of

deadenylases (such as CCR4-NOT) and is followed by mRNA instability and degradation. The mRNA translation is blocked at the initiation step or at the elongation step by inhibiting the eukaryotic initiation factor 4E (EIF4E)[229].

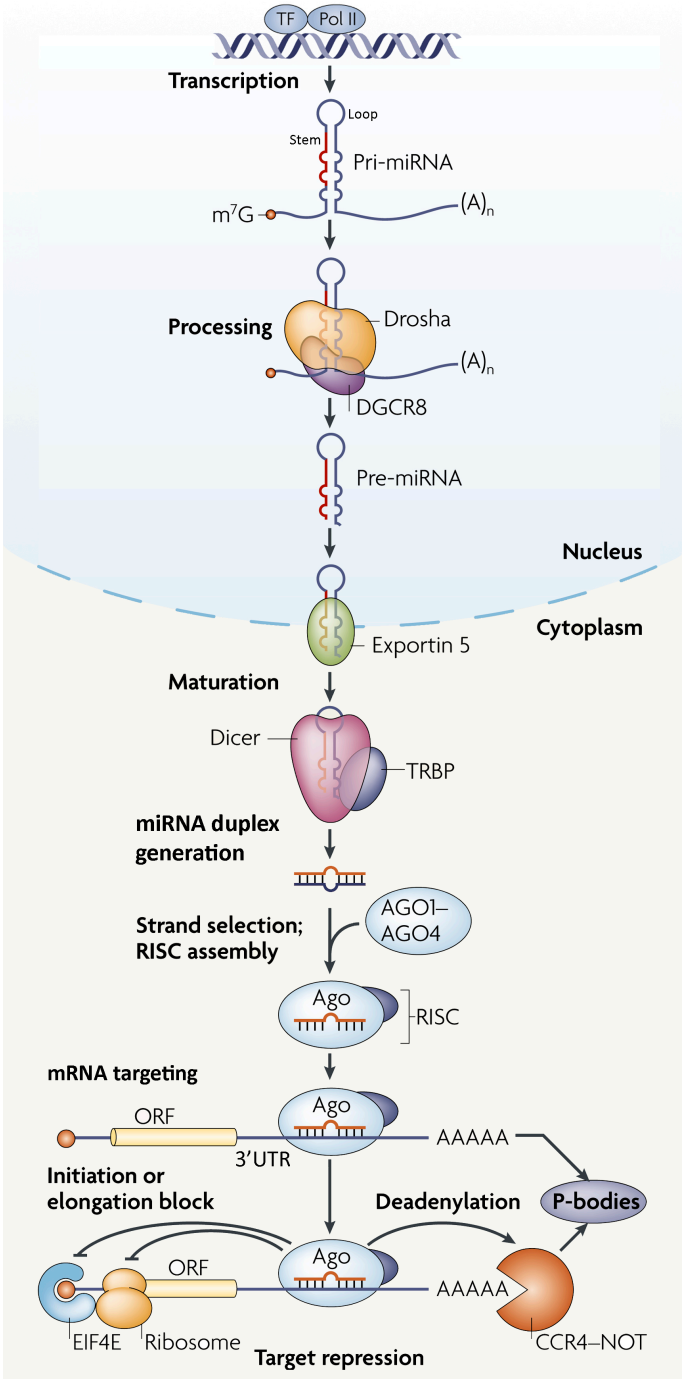

Figure 3.1 | RNA biogenesis and mechanism of action. Adapted from Inui *et al.*[229].

The mature miRNA has only a partial complementary with its target. In particular, the perfect base pairing between the seed sequence of the miRNA (from the second to the eighth nucleotide) and the seed match sequence in the mRNA 3'UTR is crucial[229]. The lack of perfect complementarity prevents the target from being sliced by the Argonaute proteins that are responsible for the "slicer" activity of the RISC. In addition, some Argonaute proteins in humans lack the catalytic residues needed for the slicer activity[230]. Actually, of the four human Argonaute proteins (AGO1, AGO2, AGO3 and AGO4), only AGO2 has demonstrable slicer activity[231,232]. There is evidence that the miRNA-silenced mRNA is directed to processing bodies (P-bodies), in which the targeted transcript is released from its inhibition or actively degraded[233].

3.4 The prediction of miRNA targets and of mRNA-targeting miRNAs

Currently, the official miRNA database miRBase lists 1527 human miRNAs (http://www.mirbase.org; Release 18)[234].

Computational methods for miRNA target prediction are very important, considering the long experimental procedures needed for verifying the direct interaction between a microRNA and its target. The task is complicated by the fact that, as mentioned above, the complementarity between miRNA:mRNA is far from being perfect. Consequently, a simple search for sequence complementarity is expected to produce many false-positive hits. Moreover, the repression of some of the several miRNA-targets is stronger than for others. Ideally, one should therefore predict not just targets, but also the expected degree of translational suppression[235].

The crucial feature for target recognition is the miRNA:mRNA pairing in at least one region. The microRNA region involved in this Watson-Crick pairing (the "seed") is located at the 5' end of the miRNA sequence (from position 1-2 to position 7-8). The corresponding site in the messenger is called "seed site" or "seed region". There are four seed types: 8mer, 7mer-m8, 7mer-A1 and 6mer, depending on the combination of the nucleotide of position 1 and pairing at position 8 (Fig. 3.2). The 6mer is the perfect 6 nt match to the miRNA seed (miRNA nucleotides 2–7). The best 7mer site, referred to as the 7mer-m8 site, contains the seed match augmented by a match to miRNA nucleotide 8. Also effective is another 7mer, the 7mer-A1 site, which contains the seed match augmented by an A at target position 1. The 8mer site

comprises the seed match flanked by both the match at position 8 and the A at position 1[236]. In addition, Watson–Crick pairing in the 3' of miRNA is known to enhance the site recognition efficacy in miRNA targets that have seed pairing[236].

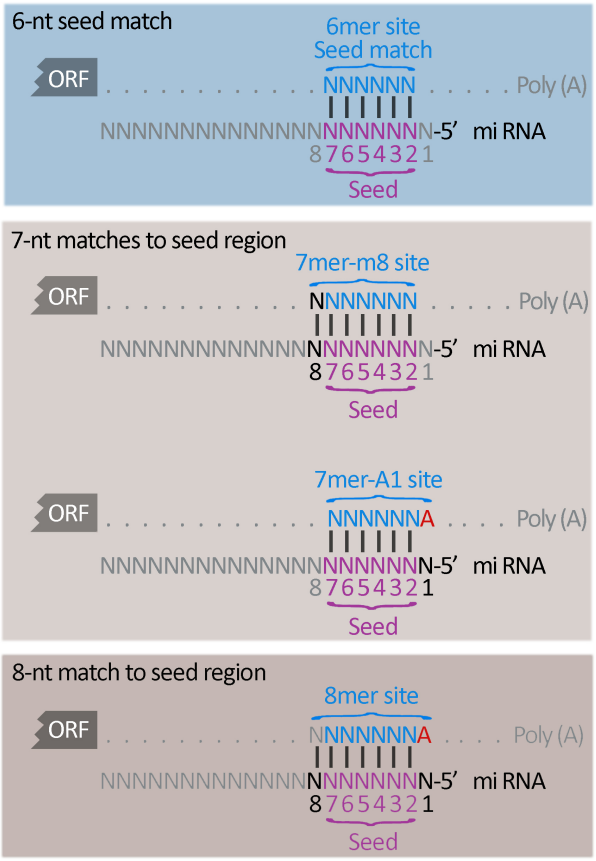

Figure 3.2 | Types of miRNA target sites. Adapted from Grimson *et al.*[241].

Although functional miRNA sites are preferentially located in the 3'UTR, seed sites in the coding sequence (CDS) and in the 5'UTR regions can also give downregulation[237,238]. Why does RISC then appear to prefer the 3'UTR? The most probable explanation is that RISC competes with other protein complexes, such as ribosomes in CDS and translation initiation complexes in 5'UTR. The 3'UTR might simply be more accessible for long-term binding than the two other mRNA regions[221]. Most miRNA target prediction studies only focus on the 3'UTR. Target genes tend to have longer 3'UTR, whereas ubiquitously expressed genes, such as house-keeping genes, have shorter 3'UTR – potentially to avoid being regulated by miRNAs[239]. Target sites are not evenly distributed within 3'UTR, but are located near both ends when the length of 3'UTR is ≥2000 nt. For shorter 3'UTRs, sites tend to be near the

stop codon[240]. Sites are not located too close to the stop codon, however, but 15–20 nt away from it[241].

Additionally, strong miRNA targets tend to have multiple target sites instead of one single site[242]. Although the general effect of multiple sites appears to be additive, miRNA targeting can also be synergistic. Two target sites within optimal distance enhance target site efficacy: the preferable optimal length is between 17 and 35 nt, but the length between 14 and 46 nt also enhances the efficacy. This co-operability is functional between the same miRNAs as well as two different miRNAs. Multiple sites involving more than two sites can also contribute to the enhancement[242]. The exact mechanism underlying the synergism remains unknown. Multiple RISC complexes bound at closely spaced target sites might cooperatively stabilize each other at the sites or possibly accelerate the regulatory process[243]. Considering the number of putative miRNA sites per mRNA can therefore significantly enhance target prediction.

miRNA families are comprised of miRNAs that have the same seed site, and are well conserved among related species. In addition, miRNA families have targets that are conserved among related species[244]. There are also species-specific miRNAs and targets, and one study showed that about 30% of the experimentally validated target genes might not be well conserved[245].

The mRNA secondary structure is very important for miRNA targeting. An effective miRNA:mRNA interaction needs an open structure on the target site to begin the hybridization reaction and the mRNA must not to be involved in any intramolecular base pairing[246]. The total free-energy change, $\Delta\Delta G$, of the binding process is therefore determined by the difference between the energy expended on opening target-site structure and that gained by forming the duplex. $\Delta\Delta G$ correlates well with the experimentally measured degree of mRNA suppression, suggesting that the dominant mechanism of miRNA target recognition is in fact simple equilibrium thermodynamics, and that $\Delta\Delta G$ determines the equilibrium constant of the binding reaction[235].

One miRNA can potentially regulate many genes; therefore, expression profiles of mRNAs might vary substantially depending on the miRNA expression levels. Many miRNAs are also expressed differently in different tissues. Consequently, it is essential that the miRNA and its putative target are co-expressed in the same cell type. Moreover, if the expression levels of the miRNA and the mRNA are negatively

correlated probably they interact each other. Filtering putative targets based on expression profile correlations is an effective approach to reduce the false-positive rate. Although the majority of miRNA targets appear to be regulated both at the mRNA and protein level, some targets only show an effect at the protein level, however[243,247].

Among the bioinformatic tools for prediction of miRNA targets and mRNA-targeting miRNAs in mammalian/vertebrates, TargetScan and Miranda are executable online tools. TargetScan often shows best performance in comparison[243]. Actually, it considers only stringent seed pairing, site number, the conservation of miRNAs and of the seed sites and site context (which includes factors that influence site accessibility). Miranda considers, on the other hand, a moderately stringent seed pairing and site number[248].

3.5 microRNAs in immunity and inflammation

3.5.1 The physiological role of microRNAs in the immune system

Haematopoietic stem cells (HSCs) reside mainly in the bone marrow and give rise to all blood cell lineages, including cells that constitute the immune system[249]. HSCs must maintain a precise balance between self-renewal and differentiation into multipotent progenitors, which subsequently give rise to both the lymphoid and myeloid branches of the haematopoietic system. Although miRNAs have been studied in other stem cell types, such as embryonic stem cells, there are currently limited data on the role of miRNAs in HSCs[250]. Several groups have carried out global miRNA expression profiling of human $CD34^+$ stem and progenitor cells and have identified certain miRNAs expressed by this cell population[251,252]. Mice deficient in ARS2, which contributes to pri-miRNA processing, have bone marrow failure possibly owing to defective HSC function[253]. These studies provide initial evidence that the miRNA pathway is important in HSC function[210].

Cells of the innate immune system, such as granulocytes, monocytes (which differentiate into myeloid-derived DCs or macrophages) and natural killer (NK) cells, provide an important first line of defence against infection. Emerging data have identified an important contribution of miRNAs to the development and function of innate immune cells. Similar to the development of innate immune cells, the development of T cells in the thymus and their activation in the periphery are also

controlled by complex protein signalling networks that are subject to regulation by miRNAs. Expression profiling of T cells has identified a broad range of expressed miRNA species and found that the expression patterns vary between T cell subsets and stages of development. Conditional deletion of Dicer in T lymphocytes results in many fewer T cells in the thymus and periphery[254]. The generation of B cells that express high-affinity antigen receptors involves two main stages: antigen-independent development in the bone marrow and antigen-dependent selection in the secondary lymphoid organs; both of which are associated with dynamic regulation by miRNAs. Dicer deficiency in B lymphocytes has been shown to diminish B cell survival and the antibody repertoire[255](see ref. O'Connell *et al.*[210]).

3.5.2 The microRNA control of signalling cascades (e.g. the TLR signalling)

Uncovering the function of individual miRNAs is challenging. First, miRNAs are frequently present as families of redundant genes, which complicates genetic dissections. Second, each miRNA has numerous putative targets that have disparate functions, with no means to decide *a priori* which one is most meaningful and thus worthy of experimental validation. Third, the degree of target downregulation imposed by miRNAs often tends to be quantitatively modest: measured at the protein level, even an overexpressed miRNA typically down-regulates most of its endogenous targets by less than 50%[247]. Therefore, most proteins should remain effective over this degree of inhibition, an argument supported by the paucity of haploinsufficient phenotypes that have been described so far. Conversely, few genes are known to have phenotypes when duplicated, perhaps mimicking the situation when miRNA-mediated regulation of a target is lost. These considerations suggest that even though most genes are predicted to be miRNA targets, only a fraction of these interactions will prove instrumental for overt biological responses and phenotypes[248]. Remarkably, however, inhibition of miRNA biogenesis (for example, through the ablation of Dicer) clearly reveals that miRNAs are essential for a wide array of biological processes, including control of proliferative homeostasis, differentiation or embryonic stemness[256].

Signal transduction pathways are prime candidates for miRNA-mediated regulation in animal cells. Signalling complexes are indeed highly dynamic and ephemeral molecular ensembles, which translate into well-established dose-dependent responses. As such, they are the ideal targets for the degree of quantitative

fluctuations imposed by miRNAs. This might enable the multi-gene regulatory capacity of miRNAs to remodel the signalling landscape, facilitating or opposing the transmission of information to downstream effectors in an effective and timely manner[257]. miRNAs targets are either positive or negative modulators of key signalling pathways[229].

MicroRNAs, emerged as immunomodulators, are important controllers of Toll-like receptor (TLR) signalling. It is well established that TLRs have important roles in detecting pathogens and in initiating inflammatory responses that subsequently prime specific adaptive immune responses during infection[258]. It has also been recognized that deregulation of this process is a hallmark of inflammatory and autoimmune diseases[259]. It is therefore important that TLR signalling pathways are tightly regulated. Actually, the signalling molecules of each TLR signalling pathway are regulated by numerous mechanisms, including physical interactions, conformational changes, phosphorylation, ubiquitylation and proteasome-mediated degradation[260]. The miRNA-mediated control of the expression of these signalling molecules — through either mRNA decay or translational inhibition — might not be as rapid as control through proteasomal degradation; however, this might be an advantage during infection, as miRNA-mediated control of mRNA levels allows for a strong initial immune response that is gradually dampened down[261].

The end result of the TLR signalling pathways is the activation of pro-inflammatory transcription factors that enhance the transcription of RNA polymerase II-sensitive genes such as those encoding cytokines, chemokines and antimicrobial enzymes. Because miRNAs are also transcribed by RNA polymerase II, it stands to reason that miRNAs themselves are targets of TLR signalling pathways[261].

Multiple miRNAs are induced in innate immune cells, with a consensus emerging that miR-155, miR-146 and miR-21 are particularly ubiquitous. There is also evidence that the expression of certain miRNAs can decrease following TLR activation. Similar to other TLR-responsive genes, miRNAs can be classified as early or late response genes: some miRNAs (e.g. miR-155) are highly induced 2 hours after treatment, whereas other miRNAs (e.g. miR-21) are induced at later times. The expression of most TLR-responsive miRNAs described so far depends on NF-κB activity. It is also important that the induction of TLR-responsive miRNAs is regulated and mechanisms are now being discovered that negatively regulate miRNA induction by TLR

signalling; for example, miR-155 in particular is subject to negative regulation by interleukin-10 (IL-10)[262].

Table 3.1 | Verified miRNA-targets belonging to the TLR signalling. Adapted from O'Neill *et al.*[261] (for references see O'Neill *et al.*[261])

Target mRNA	miRNA(s)
Receptors	
TLR4	miR-223, let-7i, let-7e
TLR3	miR-223
TLR2	miR-105
Signalling molecules	
MYD88	miR-155
MAL	miR-145
IRAK1	miR-146
IRAK2	miR-146
TRAF6	miR-146
BTK	miR-348
TAB2	miR-155
IKKα	miR-223
IKKβ	miR-199
IKKε	miR-155
Transcription factors	
NF-κB1	miR-9
FOXP3	miR-155
C/EBPβ	miR-155
PPARγ	miR-27b
p300	miR-132
Cytokines	
IL-6	let-7
TNF	miR-16, miR-125b, miR-155, miR-221, miR-579, miR-369-3
IL-10	miR-106, miR-466l
IL-12p35	miR-21
Regulators	
ACHE	miR-132
PDCD4	miR-21
SHIP1	miR-155
SOCS1	miR-155

Very recently, the ceRNA (competitive endogenous RNA) hypothesis has been proposed by Pandolfi *et al.* They hypothesize that all types of RNA transcripts (from protein coding-gene and pseudogenes to long noncoding RNAs, lncRNAs) can crosstalk through the micro-RNA binding elements (MREs), forming a large-scale regulatory network across the transcriptome that is governed by microRNA competition. According to the ceRNA hypothesis, pseudogenes and lncRNAs can compete with protein-coding genes for the same pool of microRNAs through sets of

conserved MREs. With this logic, mRNAs, usually viewed as passive targets of micro-RNAs mediated repression, possess the noncoding function of regulating the activity of microRNAs[263].

In Table 3.1, the verified targets of miRNAs in TLR signalling are summarized. The targets belong to all the molecule categories involved in the signalling cascade: receptors, signalling proteins, transcription factors, cytokines and regulators[261].

The molecular targets of miR-146a are IL-1R-associated kinase 1 (IRAK1) and TNFR-associated factor 6 (TRAF6)[264]. These proteins are important components of the myeloid differentiation primary-response protein 88 (MyD88)-dependent pathway for NF-κB activation downstream of TLR2, TLR4, TLR5, TLR7–TLR8 and TLR9, which are the same TLRs that induce expression of miR-146 in the THP-1 cell line. It was postulated that miR-146a could negatively regulate the MyD88–NF-κB signalling pathway after bacterial infection[264]. Recently, IRAK2, a kinase that is required for the persistence of NF-κB activation, has also been shown to be targeted by miR-146a[265].

Since a too strong or too prolonged innate immune response can have detrimental effects, resulting in acute and chronic inflammatory disorders, the immune system cells have to stringently employ a multilayered structure to control the immune response[266]. miR-146a belongs to this mechanism of control and, actually, it was the first identified immune system regulator in a systematic effort to find miRNAs that influence the mammalian response to microbial infection[264].

Not only in THP-1 cells, but also in human primary DCs, miR-146a production increases in response to LPS[267], whereas in B cells with latent infection with Epstein-Barr virus, miR-146a expression increases as a result of signalling of the virus-encoded latent membrane protein 1 (LMP1), which acts as a constitutively active homolog of the TNF receptor[268]. Many expression-profiling studies have demonstrated distinct patterns of miR-146 expression in various hematopoietic lineages, which suggests its involvement in the maintenance of lineage identity in lymphocyte subsets. For example, miR-146 abundance is higher in T helper type 1 cells, but lower in T helper type 2 cells relative to that in mature naive T cells[269]. In addition, miR-146 is among the most highly expressed miRNAs in regulatory T cells, and overall, the miRNA expression pattern of the regulatory T cell lineage resembles the profile of activated rather than naive T cells[270].

The miR-146a miRNA has been illustrated as one of the key molecules not only of the inflammatory response but also of oncogenesis[271]. The high expression of miR-

146a has been shown to be up-regulated in many inflammatory diseases such as osteoarthritis and rheumatoid arthritis (RA), the latter involving up-regulation following stimulation with inflammatory cytokines such as TNFα and IL1-β. Interestingly, a polymorphism in the 3'UTR of the mRNA encoding the miR-146a target IRAK1 is associated with a susceptibility to RA[272] and psoriatic arthritis[273].

An additional highly interesting paper[274] implicated loss of miRNA function with susceptibility to disease. miR-146a, along with miR-145, were pinpointed as key mediators of 5q syndrome, a subtype of myelodysplastic syndrome, one of the most common haematopoetic malignancies which progresses to acute myeloid leukaemia. 5q syndrome is defined by an isolated interstitial deletion of chromosome 5q that leads to the loss of the genes encoding miR-146a and miR-145. The authors proposed a model in which deletion of 5q results in haploinsufficiency of miR-145 and/or miR-146a. The subsequent increased expression of the miRNA target genes TIRAP and TRAF6 leads to inappropriate activation of innate immune signalling. This activation of the TRAF6-mediated signalling pathway leads to cell abnormalities and to the tendency of developing marrow failure and acute leukaemia.

Regarding miR-155 that is processed from a primary transcript, called '*BIC*', there is mounting evidence that it can negatively regulate TLR signalling pathways by targeting key signalling proteins. Inhibition of miR-155 in DCs resulted in upregulated expression of components of the p38 mitogen-activated protein kinase (MAPK) pathway[275]. TAK1-binding protein 2 (TAB2), a signalling molecule downstream of TRAF6 that activates MAPK kinases, was confirmed as a direct target of miR-155[275]. MyD88 has also been identified as a target of miR-155 in studies of miR-155 induction by *Helicobacter pylori*[276]. Furthermore, MyD88 is a target of miR-155 in foam cells, which induce the expression of miR-155 when loaded with oxidized low-density lipoprotein[277]. Analysis of both miR-155 and its Kaposi's sarcoma-associated herpesvirus (KSHV) homologue has identified IKKε as a potential target, which supports the notion that miR-155 can negatively regulate innate immune signalling[278,279]. The transcriptional co-repressor CCAAT/enhancer-binding protein-β (C/EBPβ) has been identified as another target of miR-155[280].

All together these results highlighted a negative effect on inflammation exerted by miR-155, but, in addition, miR-155 targets two negative regulators of cytokine-mediated and TLR signalling pathways: the suppressor of cytokine signalling 1 (SOCS1)[281] and SHIP1[282]. These data elucidate a role of miR-155 as a positive

regulator of immunity[266], like the studies on miR-155 knockout mice suggested. Actually, miR-155 knockout mice, the first immunologically significant miRNA-deficient animals generated, are immunodeficient, producing lower levels of antibody with impaired B-cell function and skewed Th2 responses. They also develop changes to lung tissue, with scarring that is similar to some human systemic autoimmune disorders[283,284]. Both the positive and negative aspects of these control mechanisms implicate miR-155 as a highly interesting and significant player in downstream inflammatory pathways, and further research in this area will elucidate its main role in the immune response[266].

In monocytes, macrophages and myeloid DCs, miR-155 increases substantially after exposure to a variety of inflammatory stimuli[264,267]. Direct recognition of microbial products by TLRs, particularly pathogen-associated molecular motifs derived from bacteria or viruses, leads to miR-155 upregulation. Furthermore, a plethora of immunoregulatory cytokines, such as TNF and interferons, also induce miR-155 upregulation in macrophages. Because miR-155 induction involves both the transcription factor NF-κB and the kinase Jnk it fits the profile of a typical immune-response molecule[285]. The function of miR-155 in macrophages remains an open issue. Further elucidation of miR-155 targets should show whether it is a positive or negative regulator of the macrophage inflammatory response or whether it has a more specialized task. For example, miR-155-deficient myeloid DCs have an impaired ability to trigger T cell activation after antigen presentation, which indicates its involvement in bridging innate and adaptive immunity[283].

Moreover, miR-155 was the first oncogenic miRNA ("onco-miR") to be discovered and, now, it is at the centre of much research as both a biomarker and promoter of several types of B-cell lymphoma, in particular Hodgkin's lymphoma and diffuse large B-cell lymphoma, since its expression elevated in these diseases[266].

Finally, in KSHV-infected lymphatic endothelial cells, miR-132 was shown to directly target the transcriptional co-activator p300, which is often associated with cAMP-responsive element-binding protein (cREB) and is required for the induction of antiviral genes[286]. The identification of another target of miR-132, such as the acetylcholinesterase (ACHE), a key regulator of TLR signalling, has provided links between TLRs and neuroinflammation. ACHE hydrolyses acetylcholine (an important component of the cholinergic anti-inflammatory pathway that is released by efferent vagus nerve fibres) and acetylcholine can block NF-κB nuclear translocation in

macrophages and thus attenuate TLR-induced innate immune responses. So, an increase in miR-132 levels in response to TLR stimulation will result in the repression of ACHE and increased acetylcholine-mediated negative regulation of TLR-induced signals. Indeed, LPS-treated macrophages from mice in which the 3′UTR of Ache mRNA has been deleted and therefore cannot bind miR-132 overproduce IL-6, IL-12 and TNF[287].

More recently, the TLR- responsive miRNA miR-9 was shown to directly target NF-κB1 mRNA. NF-κB1 is cleaved to form the NF-κB p50 subunit, which has an important role in transactivation of the NF-κB p65 subunit. Therefore, the finding that miR-9 can target the *NF-κB1* gene identifies a key control point for TLR signalling[288].

In conclusion, microRNAs function as efficient fine-tuners of protein expression, rather than as "on-off" switches, and they represent a mechanism to tone down the inflammatory response, which left unchecked, can have catastrophic effects[209].

Aim of the thesis

Of the estimated 30˙000 genes in the human genome, only a small fraction is activated in each cell type according to the particular stage of cell differentiation, the cell function and the environment. Gene expression is a highly specific process that allows the cell to function. Indeed, a change in the DNA code or a variation in any of the intermediate steps of gene expression process could give rise to a biological effect and/or a clinical phenotype. Single nucleotide polymorphisms (SNPs) and microRNAs represent two ways in which gene expression can be modified/regulated at two different levels.

The long pentraxin PTX3 is involved in angiogenesis, tissue remodelling and female fertility and it is an important effector and modulator of innate resistance and inflammation. PTX3, like all the members of pentraxin superfamily, is conserved in evolution from arachnids and insects to humans. This conservation is a demonstration of its crucial role in complex organisms. Indeed, a correct regulation of PTX3 expression is critical.

The purpose of this thesis was the investigation of two potential levels of PTX3 regulation: *PTX3* gene SNPs and PTX3-targeting microRNAs. SNPs are permanent transmissible variations in DNA sequence occurring in more than 1% of a population. SNPs can be associated to some clinical conditions, for example modifying the susceptibility to common complex diseases, like coronary artery disease. microRNAs inhibit the translation of specific targets at the post-transcriptional level and they could therefore be as important as transcription factors in controlling the protein content of a cell.

Hence, aims of this thesis were:

Aim 1 – to address the role of *PTX3* single nucleotide polymorphisms in modulating the susceptibility to acute myocardial infarction and PTX3 plasma levels;

Aim 2 – to evaluate the microRNA-mediated regulation of PTX3 expression.

Aim 1

Atherosclerosis, the leading cause of mortality in industrialized countries, is the principal process contributing to the pathogenesis of coronary artery disease (CAD) and acute myocardial infarction (AMI). Most CAD cases are multifactorial in aetiology, involving numerous environmental and heritable risk factors. Inflammation is a crucial component in the development, progression and disruption of the atheroma. Indeed, many of the genes that have been implicated until now in CAD are involved in innate and adaptive immunity. Thus, *PTX3* was a good candidate gene to study its involvement in atherosclerosis and in its major life threatening complication, AMI. Actually, PTX3 has cardio- and athero-protective functions and is a diagnostic and prognostic marker of cardiovascular disease.

In the present work, we addressed the eventual association of *PTX3* single nucleotide polymorphisms with PTX3 plasma levels and the risk of developing AMI in a Caucasian population.

Materials and methods

Study population

Three *PTX3* SNPs were analysed in a Caucasian population of 3245 subjects recruited by four participating units (Table 1): Istituto Clinico Humanitas (Rozzano, MI), Istituto di ricerche farmacologiche "Mario Negri" (MI), Istituto "Casa Sollievo della Sofferenza" (San Giovanni Rotondo, FG) and Ospedale "Francesco Lastaria" (Lucera, FG) in Italy.

The population was stratified into two diagnostic groups: the case group (1751 subjects) and the control group (1494 subjects). Patients of the first group had an acute myocardial infarction with ST elevation (AMI) and they presented at least one conventional coronary risk factor, such as cigarette smoking, hypertension (systolic blood pressure higher than 150 mm Hg or diastolic blood pressure higher than 85 mm Hg), diabetes mellitus (blood glucose level ≥ 126 mg/dL), hypercholesterolemia (total serum cholesterol level ≥ 200 mg/dL), dislipidemia (low density lipoprotein ≥ 130 mg/mL or high density lipoprotein ≤ 40 mg/dL or triglycerides ≥ 150mg/dL) and one relative with CAD minimum. Conversely, the control subjects did not have history of CAD, but at least one of the previously mentioned coronary risk factors. In this last group, a smaller subgroup of 362 subjects was further identified through coronary angiography that showed healthy coronaries characterized by the absence of stenosis and/or wall abnormalities. This subgroup of controls underwent coronary angiography before a cardio-surgical intervention for valvular disease (i.e. aortic or mitral regurgitation or stenosis). 1448 subjects out of 2458 provided by Istituto "Mario Negri" were from the GISSI-Prevenzione trial. The clinical data and the baseline characteristics of this population have been previously described[289]. Briefly, the GISSI-prevenzione, an open controlled clinical trial, was conducted on patients surviving beyond the hospital phase of AMI. Its objective was the evaluation of pharmacological treatments aimed to improve the profile of AMI patients, in terms of survival and quality of health. The age of all the subjects included in the case study ranged between 45 and 65 years.

Venous blood samples (5 mL in ethylenediaminetetraacetic acid) were collected from all the adult subjects that signed an informed consent to the study.

DNA extraction, sequencing and genotyping

Genomic DNA extraction was performed from frozen blood using QIAamp DNA Blood Mini Kits (Qiagen), following the manufacturer's protocol. DNA purity and concentration were evaluated using Thermo Scientific NanoDrop™ 1000 Spectrophotometer.

DNA samples of 50 cases and 50 controls recruited by Istituto Clinico Humanitas were used for the amplification of the entire *PTX3* coding sequence and of intron/exon boundaries by Polimerase Chain Reaction (PCR). PCR products were purified using the Wizard SV gel and PCR Clean-up system (Promega). For the subsequent sequencing reactions the Big Dye Terminator v1.1 Cycle Sequencing (Applied Biosystems, Foster City, CA) was used. The automated sequencing was performed on the ABI 3100 automated sequencer. The same primers were used for the amplification and the sequencing of *PTX3* exon 1 (FW: 5'-CTCCGCTCAAACTCAGCTC-3'; REV: 5'-GGTGGATATGTAGTCAGGGTTAG-3'), exon 2 (FW: 5'-AGGTCAGCTTTTACAAAGCACA-3'; REV: 5'-TAGAAGGTTGCCTGGCTTG-3') and exon 3 (FW: 5'-CGTAATGTA GGGTTTCACATAGC-3'; REV:5'-CACTGAGTTTTCAGACCTTCCC-3').

For the large-scale genotyping the 7900 Real-time PCR System (ABI) was used. Real-time PCRs were carried out in a volume of 25µl containing TaqMan Genotyping Master Mix, specific TaqMan®SNP genotyping Assays purchased from Applied Biosystems (ABI, Foster city, CA) and 20 ng of genomic DNA, according to the manufacturer's instructions. The fluorescent data files for each plate were analysed using Sequence Detection System version 3.2 (ABI, Foster city, CA). To ensure the quality of automatic allele calling, all samples were analysed in two replicates, and the concordance rate was 100%. No genotype data were missing.

PTX3 dosage

Plasma-EDTA PTX3 levels were measured by an enzyme–linked immunosorbent assay (ELISA) from Perseus Proteomics Inc. (Tokyo, Japan)[104].

PTX3 levels were measured in 704 AMI patients (from GISSI-Prevenzione trial) and 664 controls. For AMI patients, blood was collected al least 5 days after the onset of symptoms of the last event (the index event or a later event during the follow-up).

In AMI patients, PTX3 plasma levels return to normal values 125 hours after symptom onset[290].

PTX3 levels from 76 controls were excluded from the analysis because blood was sampled too close to coronarography. Moreover, PTX3 levels of 7 controls were set to missing values because they were greater than 99th centile, corresponding in our sample to a PTX3 level of 10 ng/mL. Finally, PTX3 levels from 4 AMI patients were kept out from the analysis because blood was sampled before the time needed for normalization after the last event[290]. The 5% (87/1455) of available PTX3 levels were excluded from the analysis.

Statistical analysis

Continuous variables were compared among groups using Student *t* test, ANOVA or Kruskall Wallis rank test as appropriate. Differences in percentages were assessed by Chi-square test.

The pairwise LD among the three SNPs was assessed by the correlation coefficient R^2. For each SNP, a Chi-square test was done to assess whether the observed genotype frequencies were in Hardy Weinberg Equilibrium (HWE) among controls. HWE was tested using the genhw STATA package.

Frequency distributions by genotype and by minor allele were compared across groups. Odds ratios (OR) and 95% confidence intervals (CI) were calculated comparing AMI group with the controls. A codominant genetic model has been tested.

In order to quantify the association between each SNP genotypes, diagnostic groups and PTX3 levels, a linear regression model was fitted, including age and sex as covariates. The interaction of each SNP with the diagnosis on PTX3 levels was evaluated by adding product terms to a multiple regression linear model, adjusted by age and sex.

Haplotype frequencies for rs2305619, rs3816527 e rs1840680 SNPs have been estimated by EM algorithm using UNPHASED 3.1[291]. Rare haplotypes (frequency<0.05) have been ignored.

A multivariable logistic regression model was used to determine, after adjusting for sex, age and other available clinical covariates, the effect of PTX3 level on the risk of death, cardiovascular death and reinfarction among AMI patients.

A p-value < 0.016 was considered significant to adjust for multiple testing across the three SNPs.

Statistical analysis was done using STATA 9.0 (http://www.stata.com).

Results

Association between risk of AMI and *PTX3* genetic variants

The population sample analysed in our study was composed of 3245 Italian subjects, including 1751 AMI patients and 1494 controls. Among the controls, 362 underwent coronary angiography and exhibited undamaged coronaries.

The baseline characteristics of our case study are shown in Table 2: age, sex, BMI (Body Mass Index) and lipids are described separately for AMI patients and controls. The case and control populations were very homogeneous in terms of age, whereas among AMI patients more males were present than females. As expected, control subjects had significantly higher HDL cholesterol levels and significantly lower levels of triglycerides than AMI patients. BMI and LDL cholesterol did not vary significantly between AMI and controls.

To identify *PTX3* genetic variants present in the Caucasian population, we performed a screening of the entire *PTX3* coding sequence and of intron/exon boundaries in 100 subjects of the case study (50 controls and 50 cases). The sequence analysis of amplified regions revealed the presence of three SNPs (Fig. 1). Two of them were located in the intronic sequence of *PTX3* gene (rs2305619 in intron 1 and rs2316706 in intron 2), whereas the third, rs3816527 in exon 2, was responsible for an amino acid change in position 48 (Asp48Ala), leading to a change in PTX3 primary structure.

Because the rs2305619 and rs2316706 SNPs were in complete Linkage Disequilibrium (LD, $R^2 = 1$), we performed the large-scale genotyping only testing the intronic rs2305619 and the exonic rs3816527 SNPs. A third polymorphism (rs1840680) was included in our analysis in line with previous association studies on *PTX3* genetic variants. Indeed, rs2305619, rs3816527 and rs1840680 are the three SNPs of the GAG haplotype which have been correlated with an increased susceptibility to *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* infections[107,109] and with an increased fertility[112].

Genotyping was complete for all individuals. Genotype and allele frequencies among controls of rs2305619, rs3816527 and rs1840680 SNPs are represented in Fig. 2. Information about rs2305619, rs3816527 and rs1840680 SNPs are reported in Table 3. Genotype frequencies of all the SNPs analysed resulted in Hardy-Weinberg Equilibrium (HWE) among controls (p=0.97, p=0.32, p=0.98 for rs2305619,

rs3816527 and rs1840680, respectively). The minor allele frequencies (MAF) resulted similar to the frequencies reported in HapMap. Both rs3816527 and rs1840680 SNPs were high correlated with the first polymorphism rs2305619 (R^2=0.7, R^2=0.9, respectively).

Genotype frequencies of the three analysed SNPs (Table 4) as well as haplotype frequencies were compared between AMI patients and controls or angiographically controlled individuals. None of genotypes or haplotypes was significantly associated with susceptibility to AMI.

Table 1 | Distribution of cases and controls among recruitment centres.

Institute	Tot	AMI	Controls	Controls with undamaged coronaries
Istituto Clinico Humanitas and Tor Vergata	597	299	298	268
Istituto "Casa Sollievo della Sofferenza"	94	0	94	94
Ospedale "Francesco Lastaria"	96	0	96	0
Istituto "Mario Negri"	2458	1452	1006	0
Tot	**3245**	**1751**	**1494**	**362**

Table 2 | Main clinical characteristics of the case and control populations.

		AMI (N=1751)	Controls (N=1494)	*p-value*
Sex	% male	81.8	71.8	*<0.001*
Age (years)	Mean (SD)	58.1 (7.9)	58.3 (8.1)	*0.5*
BMI (Kg/m^2)	Median (IQR)	26.4 (24.3,29.0)	26.4 (24.2,28.9)	*0.4*
LDL (mg/dL)	Median (IQR)	107.0 (85.0,132.5)	115.0 (97.0,140.5)	*0.01*
HDL (mg/dL)	Median (IQR)	40 (33,48)	54 (46,64)	*0.0001*
Triglycerides (mg/dL)	Median (IQR)	145 (107,193)	107 (78,146)	*0.0001*

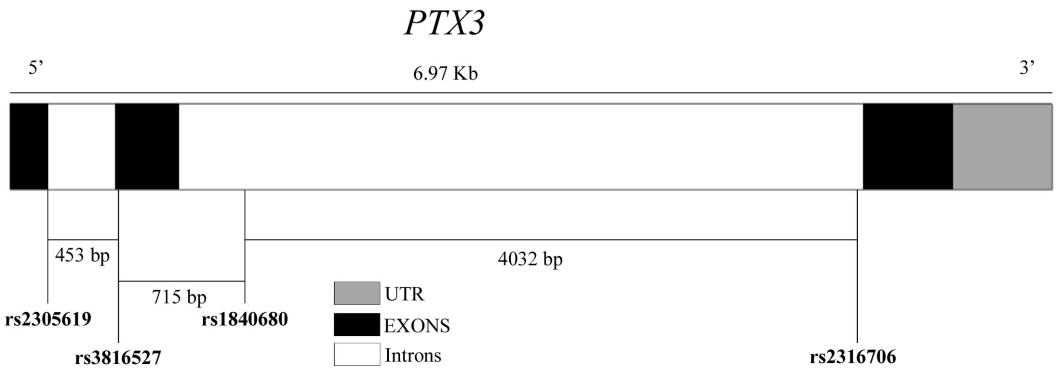

Figure 1 | PTX3 gene details. Gene map with position of SNPs typed and inter-marker distances.

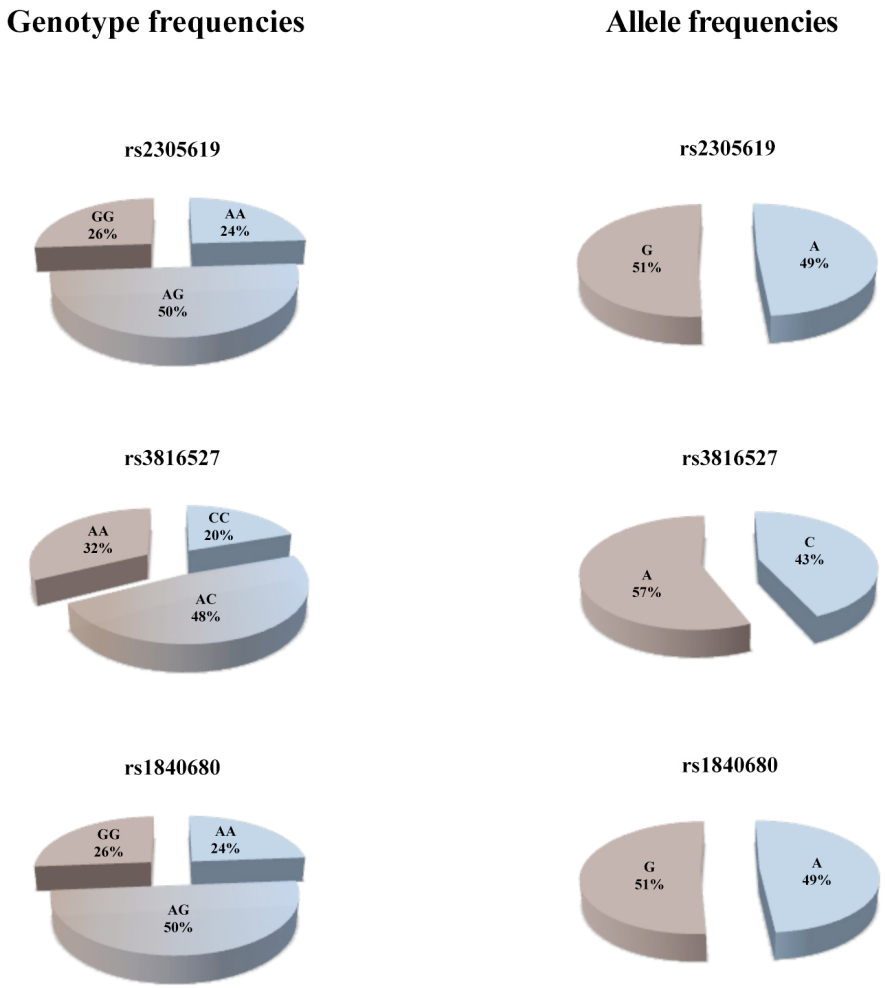

Figure 2 | Genotype and allele distribution of rs2305619, rs3816527 and rs1840680 SNPs in the group of control.

Table 3 | SNPs information.

SNPs	Position (bp)	HWE Chi-square test *(p-value)*	Minor allele	MAF	MAF HapMap	R^2
rs2305619	157154861	*0.97*	A	0.48	0.508	reference
rs3816527	157155314	*0.32*	C	0.43	0.403	0.7
rs1840680	157156029	*0.98*	A	0.48	0.491	0.9

Table 4 | Distribution of rs2305619, rs3816527 and rs1840680 SNP genotypes between AMI and controls.

SNP	Genotype	AMI N=1751	Controls N=1494	*p-value*
rs2305619	**AA**	400 (22.49%)	359 (24.03%)	*0.68*
	AG	871 (49.94%)	746 (49.93%)	
	GG	473 (27.12%)	389 (26.04%)	
rs3816527	**CC**	310 (17.74%)	292 (19.58%)	*0.39*
	AC	864 (49.46%)	714 (47.89%)	
	AA	573 (32.80%)	485 (32.53%)	
rs1840680	**AA**	395 (22.75%)	355 (23.76%)	*0.72*
	AG	867 (49.94%)	746 (49.93%)	
	GG	474 (27.30%)	393 (26.31%)	

Association between PTX3 plasma levels and *PTX3* genetic variants

Next, we evaluated the possible effect of the three SNPs on PTX3 plasma levels in the two diagnostic groups. In literature, it has been reported that PTX3 levels in blood are associated with age and sex[290,292]. In our case study a significant increase of PTX3 concentrations has been found in association with age (β=0.02; p=0.002), but not with sex. PTX3 concentrations were measured in 1368 subjects, including 704 AMI patients (from GISSI-Prevenzione trial) and 664 controls. PTX3 plasma levels (mean ± SD) resulted significantly higher in AMI patients (3.61 ± 2.43 ng/mL) than in controls (2.93 ± 1.52 ng/mL, p=0.0001). For AMI patients, the distribution of PTX3 levels according to the time of blood collection is reported in Fig. 3. The blood was sampled in a period spanning from 5 to 733 days from the last event and three ranges of time were identified: the first spanning from 5 to 63 days, the second from 64 to 184 days and the third from 185 to 733 days. For each time range, the mean of PTX3

concentration was measured. In the first range, PTX3 levels (4.18 ng/mL) were significantly different ($p<0.0001$) from PTX3 values in the second (3.31 ng/mL) and in the third range (3.33 ng/mL). No differences were found in the distribution of *PTX3* genotypes among the three groups generated according to the time of blood collection.

The distribution of PTX3 plasma levels among rs2305619, rs3816527 and rs1840680 genotypes is shown in Table 5. In control patients, PTX3 levels were significantly different among genotypes for all the three SNPs. PTX3 levels in AMI patients showed the same trend, although differences were not significant. In particular, the carriers of the AA genotype at the rs2305619 SNP had the higher amount of PTX3 in the blood compared to the AG and GG carriers. The same data were obtained for the AA genotype carriers at the intronic polymorphism rs1840680 and for the CC genotype carriers, in controls, and the AC genotype carriers, in AMI patients, at the exonic polymorphism rs3816527.

Fitting a linear regression model, adjusted by age and sex, both the genotype and the diagnosis (AMI vs controls) resulted significantly associated to PTX3 plasmatic levels for all the three SNPs ($p=0.0083$, $p=0.0347$, $p=0.0032$, respectively for rs2305619, rs3816527 and rs1840680). PTX3 genetic variants did not associate with the diagnosis, suggesting the presence of a correlation between PTX3 levels and *PTX3* SNPs that is independent of the pathological condition (Fig. 4).

Moreover, the possible association between PTX3 plasma levels and haplotypes in the entire population (AMI patients and controls) was evaluated. Like each individual SNP, the haplotypes were associated with different PTX3 plasma levels (overall p-value=0.01, Table 6). Additive values estimated the change in the expected value of PTX3 plasma levels due to a given haplotype. These additive values were small but significant. AAA (for rs2305619, rs3816527 and rs1840680, respectively) and ACA haplotypes were associated with an increased value of PTX3 levels when compared to the most common GAG haplotype that was considered as reference. The ACA haplotype (frequency = 42%) was associated with an estimated increase in PTX3 plasma levels of 0.05 ng/mL, whereas for the AAA haplotype (frequency = 5%) this estimated increase was of 0.08 ng/mL compared with the GAG haplotype (frequency = 52%).

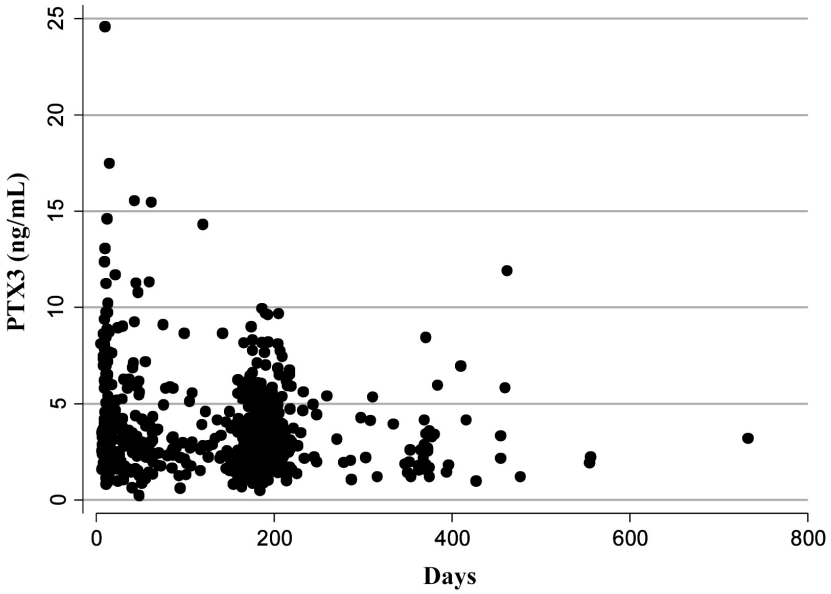

Figure 3 | Distribution of PTX3 plasma levels in AMI patients according to the time of blood collection after the last event. For 704 AMI patients (from GISSI-Prevenzione trial), PTX3 plasma levels (ng/mL) were measured at different time points, from 5 to 733 days, after the last event. Three main time ranges can be identified: the first from 5 to 63 days, the second from 64 to 184 days, the third from 185 to 733 days.

Table 5 | PTX3 plasma levels distribution among SNP genotypes in AMI patients and controls.

		IMA (N=704)		Controls (N=664)	
			PTX3 (ng/mL)		PTX3 (ng/mL)
SNP	**Genotype**	**N**	**Mean (SD)**	**N**	**Mean (SD)**
rs2305619	**AA**	154	3.84 (2.58)	152	3.22 (1.60)
	AG	344	3.67 (2.54)	352	2.84 (1.49)
	GG	206	3.32 (2.11)	160	2.83 (1.47)
	p-value		*0.08*		*0.02*
rs3816527	**CC**	118	3.56 (2.07)	126	3.22 (1.54)
	AC	344	3.80 (2.74)	331	2.90 (1.54)
	AA	242	3.36 (2.10)	207	2.79 (1.46)
	p-value		*0.15*		*0.02*
rs1840680	**AA**	154	3.89 (2.60)	149	3.25 (1.61)
	AG	340	3.66 (2.54)	355	2.83 (1.49)
	GG	210	3.31 (2.10)	160	2.83 (1.47)
	p-value		*0.06*		*0.009*

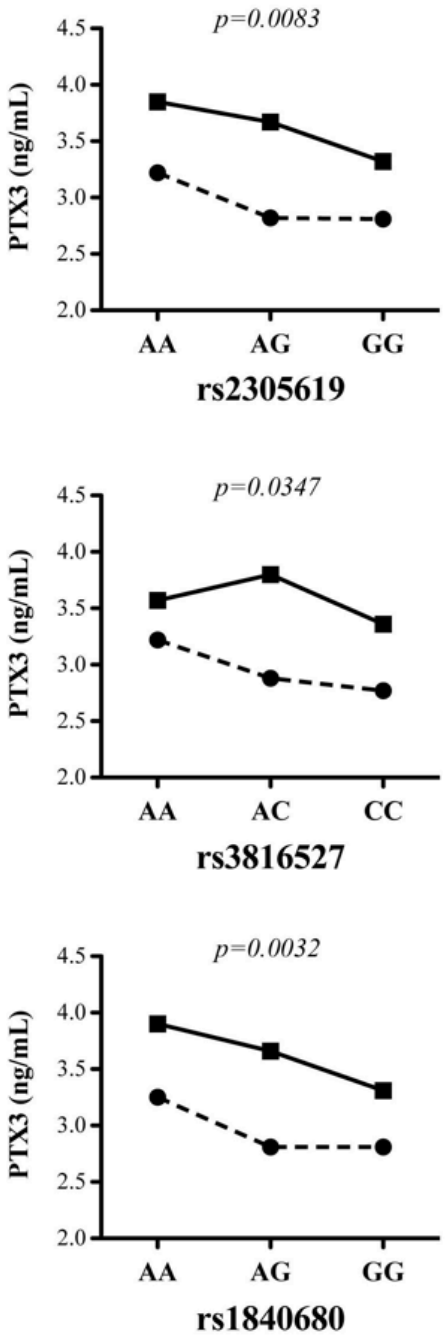

Figure 4 | Interaction plot of the estimated PTX3 mean levels, adjusted by sex and age, according to diagnosis by rs2305619, rs3816527 and rs1840680 genotypes. In each graph, the estimated mean of PTX3 levels (ng/mL) in the two diagnostic groups (—■— AMI patients, --•-- controls) according to genotypes is represented.

Table 6 | Association between common haplotypes of SNPs rs2305619, rs3816527 and rs1840680 and PTX3 levels.

Haplotype	Count	Frequency	Additive value (ng/mL)	95% CI (ng/mL)
GAG	1427	0.52	reference	reference
ACA	1162	0.42	0.05	0.009-0.08
AAA	138	0.05	0.08	0.009-0.158

Association between PTX3 plasma levels and prognosis

Next, we assessed the role of *PTX3* genetic variants in the prognosis of AMI patients in a retrospective analysis. Among 1448 AMI patients from the GISSI-Prevenzione trial, 120 deaths (8.29%), 32 cardiovascular deaths (2.21%) and 64 reinfarctions (4.42%) have been counted. Blood for PTX3 dosage was available only for 68 out of 120 deaths, 19 out of 32 cardiovascular deaths and 32 out of 64 reinfarctions. In patients with en event occurring over 3 years after the index event the age was significantly higher ($p<0.0001$ for death, $p=0.002$ for cardiovascular death, $p=0.02$ for reinfarction) than in event-free subjects. Cardiovascular deaths were significantly more frequent among females ($p=0.03$) than in males. Other clinical covariates as well as SNP genotypes were not associated with events. After an adjustment by age and sex, PTX3 levels correlated with the risk of death ($p=0.02$, OR=1.10, 95% CI: 1.10-1.20), whereas the association was not significant for cardiovascular death ($p=0.09$, OR=1.12, 95% CI: 0.98-1.27) and reinfarction ($p=0.67$, OR=1.03, 95% CI: 0.90-1.18). The distribution of the events was not statistically different amongst the three groups identified according to the time of blood collection (Fig. 3).

Discussion

In this study, we examined the role of *PTX3* genetic variations in the susceptibility to AMI. *PTX3* gene encodes for the long pentraxin PTX3, a molecule involved in the inflammatory response and associated with cardio- and athero-protective functions. Data obtained in different *in vivo* models showed that PTX3 is involved in modulating inflammation in AMI and atherosclerosis[56,85]. Moreover, it represents a useful diagnostic and prognostic marker in cardiovascular pathology[83].

Despite its strong evolutionary conservation, recent studies have reported the presence of a certain number of SNPs in the *PTX3* gene. Interestingly, some of these polymorphisms, especially when associated in particular haplotypes, have been related to clinical conditions, including infections and fertility[107,109,112].

Multiple genetic and/or environmental factors are supposed to play a role in conferring variable susceptibility to AMI. The association study reported here reveals that 3 common *PTX3* polymorphisms do not have a significant effect in modulating the risk of AMI in the Caucasian population.

However, *PTX3* SNPs, as well as the haplotypes corresponding to SNPs rs2305619 to rs1840680, are associated with different levels of PTX3 in the blood. Actually, in our case study the most frequent GAG haplotype is associated with the lower value of plasma PTX3 compared to the ACA (+0.05 ng/mL) and AAA (+0.08 ng/mL, overall p-value = 0.01) haplotypes. The GAG haplotype has been previously associated with a protective effect against pulmonary tuberculosis in West Africans[107] and *Pseudomonas aeruginosa* colonization in Italian cystic fibrosis patients[109]. Moreover, May *et al.*[112] associated the GAG haplotype not only with an increased fertility, but also with an increased *ex-vivo* LPS-induced PTX3 production by whole-blood cells in a population from Ghana. The apparent conflict of our data with that of May and coworkers can be explained by genetic background heterogeneity (with different populations having different genetic variants affecting PTX3 levels) and by the conditions of PTX3 production taken into account. Actually, May *at al.* induced the *ex-vivo* production of PTX3 by stimulating whole-blood cells with LPS, whereas our data concern the basal PTX3 plasma levels. In particular, in this study PTX3 values have been excluded from the analysis when plasma for PTX3 dosage have been sampled too close to the coronarography, in controls, or before the time for normalization after the last event in AMI patients.

The mechanism underlying the modulation of PTX3 plasma levels by *PTX3* SNPs has to be clarified yet. It is possible that the *PTX3* polymorphisms rs2305619, rs3816527 and rs1840680-belong to a larger haplotype block than the one considered here and they could be in linkage with a genetic variant located in a regulatory region, possibly the *PTX3* promoter. We envision that, in such a position, a polymorphism can affect the binding of a transcription factor to its consensus binding site, modifying the PTX3 expression level. It cannot completely be ruled out that *PTX3* SNPs are related to higher plasma levels of a functionally less active protein. Actually, the second SNP studied here (rs3816527) causes an amino acid change (Asp48Ala) in a strategic position of PTX3 primary structure. This substitution can potentially interfere with the N-terminal-mediated binding of PTX3 to its ligands. Moreover, the amino acid 48 is located between the two cysteine residues (in position 47 and 49, respectively) involved in the formation of inter-chain disulfides required for the tetrameric arrangement of four PTX3 protomers[2]. Despite a different isoelectric point (pI) between the polar aspartic acid and the non-polar alanine (pI_{Ala}=6.11, pI_{Asp}=2.85), the pI of PTX3, calculated using the EXPASY bioinformatics tool,[293] is quite similar comparing the protein carrying the aspartic acid or the alanine residue in position 48 (4.90 vs 4.94, respectively). The steric hindrance due to the side-chain of the two residues is certainly different between the two PTX3 isoforms and, therefore, could affect PTX3 activity.

It is likely that different levels of PTX3 in the blood depending on genetic variants have an influence on the outcome of specific infections. Indeed, it has been shown that the GAG/ACA haplotypes are differently associated with susceptibility to *Mycobacterium tuberculosis*[107] or *Pseudomonas aeruginosa* colonisations[109], even if in these two studies the authors did not address a correlation between PTX3 levels and *PTX3* haplotypes. It is conceivable that haplotypes affecting PTX3 levels are not relevant for the risk of developing AMI.

In our case study, the mean value of circulating PTX3 in the controls is 2.93 ± 1.52 ng/mL. This value is significantly different from PTX3 levels in AMI patients (3.61 ± 2.43 ng/mL p= 0.0001), even if blood was sampled after at least 5 days from the last event, the time needed for normalization of PTX3 concentration[290]. For the group of AMI patients, we analysed the distribution of PTX3 plasma levels in the three ranges of time identified after the last event. Up to three months after the last event, the mean of PTX3 plasma concentration is higher (4.18 ng/mL) than in a later

period (3.32 ng/mL, from three months to two years), in which PTX3 plasma levels reach values quite similar to that measured in healthy subjects. These data suggest that the high PTX3 levels, measured from 5 to 64 days after the last event, are likely responsible for the significant difference of PTX3 plasma levels observed between AMI patients and controls in our population. These results suggest that in AMI patients PTX3 plasma levels return, in few days after the onset of symptoms, to a quite normal value that remains higher, than in healthy subjects, for at least three months.

Moreover, our data confirm the already described prognostic value of PTX3 plasma levels in AMI patients. Latini *et al.* demonstrated that PTX3 is an independent predictor of 3-months mortality, when compared with other biomarkers considered reliable predictors of death in patients with AMI[91]. The retrospective analysis conducted in this study on AMI patients from GISSI-Prevenzione trial reveals a correlation between PTX3 plasma levels and all-cause death.

In this study, we provide evidence of a strong association between *PTX3* genetic variants and PTX3 plasma levels, but we did not observed a correlation between *PTX3* SNPs, responsible for a different baseline PTX3 concentration in the blood, and susceptibility to AMI (Fig. 5). A similar set of data has been obtained for another member of the pentraxin superfamily, the C-reactive protein. CRP, like PTX3, binds LDL[16] and is present in the atherosclerotic plaque[17]. Several epidemiological studies of coronary heart disease (CHD) have reported an association of increased basal CRP levels and CHD risk[30,31]. Altogether these observations suggested the possible causal role of CRP in CHD. Several SNPs have been identified in the *CRP* gene. Independently or combined in haplotypes, part of these SNPs was associated with different CRP baseline levels. Actually, a meta-analysis of genetic studies involving a new Bayesian method (enabling integration of information across studies that had typed a partially overlapping set of *CRP* SNPs) has provided evidence of four functional SNPs at the *CRP* locus that influence its circulating concentration[40]. A debate about the relationship between the genetically determined CRP blood levels and the risk of CHD has been opened for years. Randomized treatment trials specific to CRP have not yet been done in relation to cardiovascular outcomes. Therefore, genetic studies based on the "Mendelian randomisation" approach have been used to evaluate the causal relevance of CRP itself to CHD. Data obtained from large case

studies suggest that CRP concentration itself is unlikely to be even a modest causal factor in CHD[39,42].

Given our results on PTX3 and evidences on CRP in relation to CHD risk, we envision a similar situation for PTX3 and AMI risk. Actually, this study highlights the role of *PTX3* SNPs in modulating PTX3 baseline levels that, after a peak, remain higher in patients that underwent an AMI than in healthy subjects. When we evaluated the association of *PTX3* SNPs with the risk of AMI, we did not obtain a significant correlation. Indeed, PTX3 is a marker of atherosclerosis and correlates with the risk of cardiovascular events, but genetically determined high PTX3 levels do not seem to increase the susceptibility to AMI.

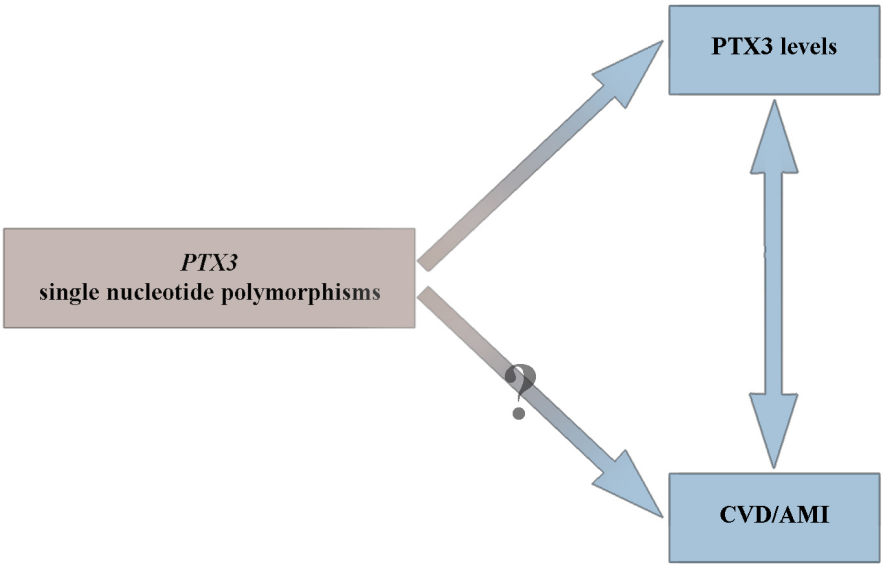

Figure 5 | Schematic representation of results obtained. *PTX3* single nucleotide polymorphisms (rs2305619, rs3816527 and rs1840680) correlate with different levels of PTX3 in the blood. High PTX3 plasma levels are observed in CVD/AMI patients. The candidate gene association analysis performed here did not reveal an association between *PTX3* SNPs and the risk of developing AMI.

Aim 2

As new regulators of gene expression, microRNAs have a great impact on protein expression of specific targets. As all “conventional” genes, their expression can be regulated in response to stimuli involved in almost all physiological and pathological processes, including inflammation. Actually, the up-regulation of a single miRNA or of a miRNA set can switch off a complete signalling pathway, by targeting receptors, adaptors, signalling molecules, transcription factors and regulators.

PTX3 production is induced in different cell types by different stimuli and we assessed whether its expression could be regulated at the post-transcriptional level by specific miRNAs.

Materials and Methods

Cell cultures and reagents

8387 human fibrosarcoma cells (ATCC) were cultured at 37ºC with 5% CO^2 in RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics. HEK293T, Human Embryonic Kidney 293 cells stably and constitutively expressing the SV40 large T antigen (ATCC), were cultured at 37ºC with 5% CO^2 in DMEM supplemented with 10% FBS, 2 mM L-glutamine and antibiotics.

RPMI 1640 and DMEM medium, 200 mM Ultraglutamine-1 (100x), 10000 U/mL Penicillin-Streptomycin solution (100x) and FBS were purchased from Lonza. Recombinant human cytokines were obtained from PeproTech (Neuilly-Sur-Seine, France). LPS from *Escherichia coli* serotype R515 (RE) (~99.9% purity) was obtained from Alexis.

Constructs generation and Luciferase Reporter Assay

The pBlueScript vector containing the entire human *PTX3* 3' untranslated region (3' UTR) was used as DNA template for performing a PCR with the M13 primer (5'-TGTAAAACGACGGCCAGT-3') and the following primer containing 8 additional nucleotides at the 5'-end, six of which (underlined) give rise to a SpeI restriction site (5'-GAACTAGTATGTTGTGAAACTCCACTTG-3'). The *PTX3* 3'UTR was excised by SpeI-XhoI digestion and subcloned in SpeI-XhoI-digested psiCHECK-2 vector (Promega, Rluc-PTX3). The ERp18 3' UTR was entirely cloned in psiCHECK-2 vector (Rluc-ERp18) by PCR cloning from a healthy donor genomic DNA (primer FW: 5'-GGGTGATGCCTTCAGAAAGA-3'; primer REV: 5'-CCCTCCCTCCTTTTCCAATA-3'). Site-directed mutagenesis reactions were conducted using the QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies (USA) in accordance with data sheet instructions. The following constructs were generated with mutated (underlined region) oligonucleotides: Rluc-mut-9-PTX3 (FW: 5'-CTCCACTTGAAGCGGGGGAAAGAAACTCACACT-3'; REV: 5'-AGTGTGAGTTTCTTTCCCCCGCTTCAAGTGGAG-3'), Rluc-mut-29-PTX3 (FW: 5'-CTAGACTTTATGCCAGCCGCCTTTCAGTTTAATGC-3', REV: 5'-GCATTAAACTGAAAGGCGGCTGGCATAAAGTCTAG-3'), Rluc-2614-PTX3 (FW: 5'-TATGCCATGGTGCTTTTAGTTTAATGCTGTGTCTCTG-3'; REV: 5'-CAGAGACACAGCATTAAACTAAAAGCACCATGGCATA-3'), Rluc-mut29-

rs2614-PTX3 (FW: 5'-ATGCCAGCCGCCTTTTAGTTTAATGCTGTGTC-3'; REV: 5'-GACACAGCATTAAACTAAAAGGCGGCTGGCAT-3'), Rluc-mut-181-ERp18-8mer (GTCAACTTGTCATTGCGCCGAAAGAATGAAACCTTC-3'; REV: 5'-GAAGGTTTCATTCTTTCGGCGCAATGACAAGTTGAC-3'), Rluc-mut-181-ERp18-8/7mer (FW: 5'-GATGAATTGTAACATGCGGCTGCCCCTTCTTTCAT-3'; REV:5'-ATGAAAGAAGGGGCAGCCGCATGTTACAATTCATC-3'). All the generated clones were sequenced and subsequently used in the Luciferase Reporter Assay.

pGV-B2 plasmid expressing firefly luciferase protein under the control of human *PTX3* promoter was obtained as previously reported[59]. pRL-TK vector, that expresses renilla luciferase gene under an eukaryotic constitutive promoter (HSV-thymidine kinase promoter), was used as internal control.

For the Luciferase Reporter Assay, HEK293T cells were plated in 24-well plates in DMEM supplemented with 10% FBS, 2 mM L-glutamine and without antibiotics to avoid possible interference with lipofectamine 2000 (Invitrogen), used for cell transfection. 8 x 10^4 cells were plated in each well 24 h before transfection. To study the direct interaction miR:3'UTR, cells were co-transfected with 100 ng of psiCHECK-2 vector (Rluc construct) together with miRNA precursors or miRNA negative control (mimics, from Ambion) to reach 100 nM as final concentration. To investigate the activity of miRNAs on the signalling cascade upstream *PTX3* promoter activation, cells were co-transfected with 50 ng of pGV-B2, 50 ng of pRL-TK and pre-miR or miRNA scramble to reach a final concentration of 100 nM and cells were stimulated or not with TNFα or IL-1β (both obtained from Peprotech). After 24 hours from transfection, cells were lysed and both firefly and renilla luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega). The enzymatic activities of the luciferases were quantified using a MultiDetection Microplate Reader Synergy 2 luminometer (BioTek). The use of psiCHECK-2 vector enables the detection of changes in the expression of the target gene (3'UTR) fused to a primary reporter gene (renilla luciferase, R-luc). The second reporter gene (firefly luciferase, Luc) is used as an internal control, allowing for the normalization of R-luc expression. Results obtained from R-luc constructs and mimics co-transfection are expressed as mean ± SEM of the ratio between renilla luciferase and firefly control luciferase activities (RLU) adjusted to 1. Each experiment was conducted in triplicate.

Results obtained from pGV-B2, pRL-TK and mimics co-transfecion are expressed as mean ± SEM of the ratio between firefly luciferase and renilla control luciferase activities (RLU) adjusted to 1. Each experiment was conducted in duplicate.

8387 Transfection

Mimic transfection was conducted in 24-well tissue culture plates. 24 h before transfection, 8 x 10^4 8387 cells were plated in complete medium without antibiotics. Complexes of lipofectamine 2000 (Invitrogen) and pre-miR or miR-control (Ambion) were assembled in Opti-MEM medium (Invitrogen) and added to each well, reaching a mimic final concentration of 100 nM. Co-transfection of mimics and siRNA molecules (Dharmacon) was performed at a final concentration of 50 nM each. After the over-night transfection, medium containing lipofectamine was removed to avoid cell death. Cells were stimulated or not with 20 ng/mL TNFα or IL-1β (both from Peprotech) in RPMI supplemented with 1% FBS. After 8 and 24 h stimulation, cells free supernatants were collected for subsequent PTX3 measurement. Each experiment was conducted in duplicate.

To assess siRNA activity on Erp18 expression, 3x10^5 8387 cells were pleated one day before transfection in a 6-well plate and siR-Erp18 (ON-TARGET plus SMART pool siRNA, Dharmacon) or siR-control (ON-TARGET plus Non-targeting siRNA, Dharmacon) were transfected using lipofectamine 2000 reagent (Invitrogen) at a final concentration of 100 nM. After 24 h, cells were collected and lysed with Trizol or Protein Lysis Buffer and mRNA or protein quantified by qRT-PCR or Western Blot analysis, respectively. 2 independent experiments were conducted.

PTX3 dosage

PTX3 protein released by 8387 cells was quantified from cell free supernatants by previously described home-made-ELISA[290]. In brief, 96-well ELISA plates (Nunc) were coated with 100 µg of anti-PTX3 monoclonal antibody (mAb) MNB4 and incubated overnight at 4°C. Nonspecific binding sites were saturated with 300 µL of 5% dry milk (2 hours at 37°C), prior the addition of purified human recombinant PTX3 standards (from 75 pg/mL to 2.4 ng/mL) and unknown samples diluted in PBS (Cambrex BioScience) and 2% bovine serum albumin (Sigma). After 2 h at 37°C, 100 ng of biotin-conjugated rabbit polyclonal anti-PTX3 were added to each well. Plates were incubated for 1 h at 37°C and streptavidin-peroxidase was then added, followed

by the addition of the TMB liquid substrate system (Sigma). After each step, plates were extensively washed with PBS (Cambrex Bio Science) containing 0.05% Tween 20 (Merck), also used for reagent dilution.

SDS –PAGE and Western Blotting

For immunoblot experiments, transfected 8387 cells were lysed in Protein Lysis Buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM PMSF, 1% Triton X-100 and a cocktail of protease inhibitors (cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche Applied Science, Canada). Protein lysates were centrifugated at 16.000xg for 10 min at 4°C and conserved at 4°C to prevent proteolysis. The protein content was determined using the Coomassie reagent (Thermo Scientific) in accordance with data sheet instructions. 35 μg of total proteins from each sample were subjected to electrophoresis in denaturing conditions on 12,5% SDS-PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad). Following the transfer, the membranes were blocked in 5% dry milk for an hour and then incubated overnight with the primary antibody at 4°C. The following day, the membranes were washed three times with 1x PBS/Tween 20 0.1% and probed with the secondary antibody (one hour incubation). Anti-ERp18 rabbit pAb (Abcam Inc.), for the detection of ERp18, and anti-Vinculin mouse mAb (Sigma-Aldrich), for Vinculin detection, were used at dilution of 1:500 and 1:5000, respectively, in 5% milk. The secondary antibodies were horseradish peroxidase-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG (GE Healthcare Bio-Sciences AB). ECL reagents (from Millipore) were used in accordance with data sheet instructions. Image J software from NIH (National Institutes of Health, Bethesda, MD) was used for gel band quantitative densitometric analysis. Selected bands were quantified on their relative intensities. Data are expressed as mean ± SEM of the ratios between ERp18 and Vinculin band intensities (AU) adjusted to 1.

Real time RT-PCR (RT-qPCR)

The expression of Erp18 gene was quantified by RT-qPCR. Total RNA of 8387 cells transfected with siR-Erp18 or siR-control was extracted using the TRIzol method according to the manufacturer's instructions (Invitrogen). RNA purity and concentration were evaluated using Thermo Scientific NanoDrop™ 1000 Spectrophotometer. 1 μg of total RNA from each sample was reverse transcribed

using the high capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. Erp18 gene expression was quantified by RT-qPCR performed in duplicate from 20 ng cDNA in the presence of the SYBR green PCR Master Mix (Applied Biosystems) and 400 nM of the specific oligonucleotides (FW:5'-GGTAAATCTTGAGGATGAAGAGGA-3'; REV:5'-TGTTTCTTTCTGAAGGCATCAC-3'). RT-qPCR data were analysed with SDS 2.2.1 software and normalized on the expression of GAPDH expression (FW: 5'-AACAGCCTCAAGATCATCAGC-3'; REV: 5'-GGATGATGTTCTGGAGAGCC-3'). RT-qPCR reaction was conducted using the Applied Biosystems 7900HT Fast Real-Time PCR System. Thermal cycler conditions were the following: 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s.

Statistical Analysis

Data are expressed as means ± SEM. Statistical changes in luciferase expression and in PTX3 production were determined using the one-way ANOVA with α set to 0.05 according to the Dunnett test. In the densitometric analysis of Western blots, statistical changes were determined performing a Student's *t* test. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; ns: $p > 0.05$.

Reesults

miR-9 and miR-29 affect PTX3 production via direct control at post-transcriptional level

In order to identify microRNAs potentially involved in the regulation of PTX3 expression, we analysed the human PTX3 3' UTR using TargetScan[294] and MiRanda[295] software. As shown in Fig. 6A, the human PTX3 mRNA presents well conserved 7-mer long seed regions for miR-9 and miR-29, located 24 and 204 bp downstream the stop codon, respectively. Both seed regions were evolutionarily conserved in mammals, but not in birds. (Fig. 6B). The ability of miR-9 and miR-29, which is a family composed by three members (miR-29a, miR-29b, miR-29c), to directly interact with the PTX3 3' UTR, was then evaluated in a luciferase-based reporter assay. HEK293T cells were transiently co-transfected with the reporter construct containing the renilla luciferase coding sequence fused to the whole human PTX3 3' UTR and 100 nM pre-miR-9 or pre-miR-29abc. As shown in Fig. 6C, miR-9 and miR-29abc reduced renilla luciferase activity by approximately 50% in comparison to the scramble control. When we mutated the miR-9 seed region on PTX3 3' UTR, the luciferase expression was restored, attesting the specificity of miR-9 base-pairing with PTX3 3' UTR. The same was also demonstrated for all the members of the miR-29 family, since the mutagenesis of miR-29 seed region reverted the inhibitory activity of miR-29abc.

These data suggest that miR-9 and miR-29 display a strong regulation of PTX3 mRNA directly acting on the target sites predicted by bioinformatic tools.

The human 8387 fibrosarcoma cell line constitutively produce PTX3 and upregulates its production in response to TNFα and IL-1β (Fig. 7 and ref. Doni *et al.* [59]). To test whether miR-9 and miR-29 family members affected PTX3 production at the protein level, 8387 cells were transfected with pre-miR-9 or pre-miR-29abc and PTX3 production was analysed by ELISA[290,296]. As shown in Fig. 8, miR-9 and miR-29 family members significantly down regulated PTX3 production compared to control cells at the basal level and after stimulation with TNFα and IL-1β. Notably, in basal conditions 8387 cells transfected with pre-miR-9 produced half amount of PTX3 than mock cells.

These results demonstrate that miR-9 and miR-29 operate a negative regulation on PTX3 at the post-transcriptional level affecting the protein production.

The *PTX3* gene presents the single nucleotide polymorphism (SNP) rs2614C>T 3 bp downstream the miR-29 seed region (Fig. 6A). The Haplotype Mapping Project database reported a frequency for the C allele of 99% in European and 90% in Sub Saharan African subjects, respectively. When the two allelic variants were tested for miR-29 activity in luciferase assay, results showed a similar effect of miR-29abc on the two 3' UTR PTX3 sequences (data not shown), indicating that this SNP is not interfering with the regulatory role of miR-29abc on PTX3 production.

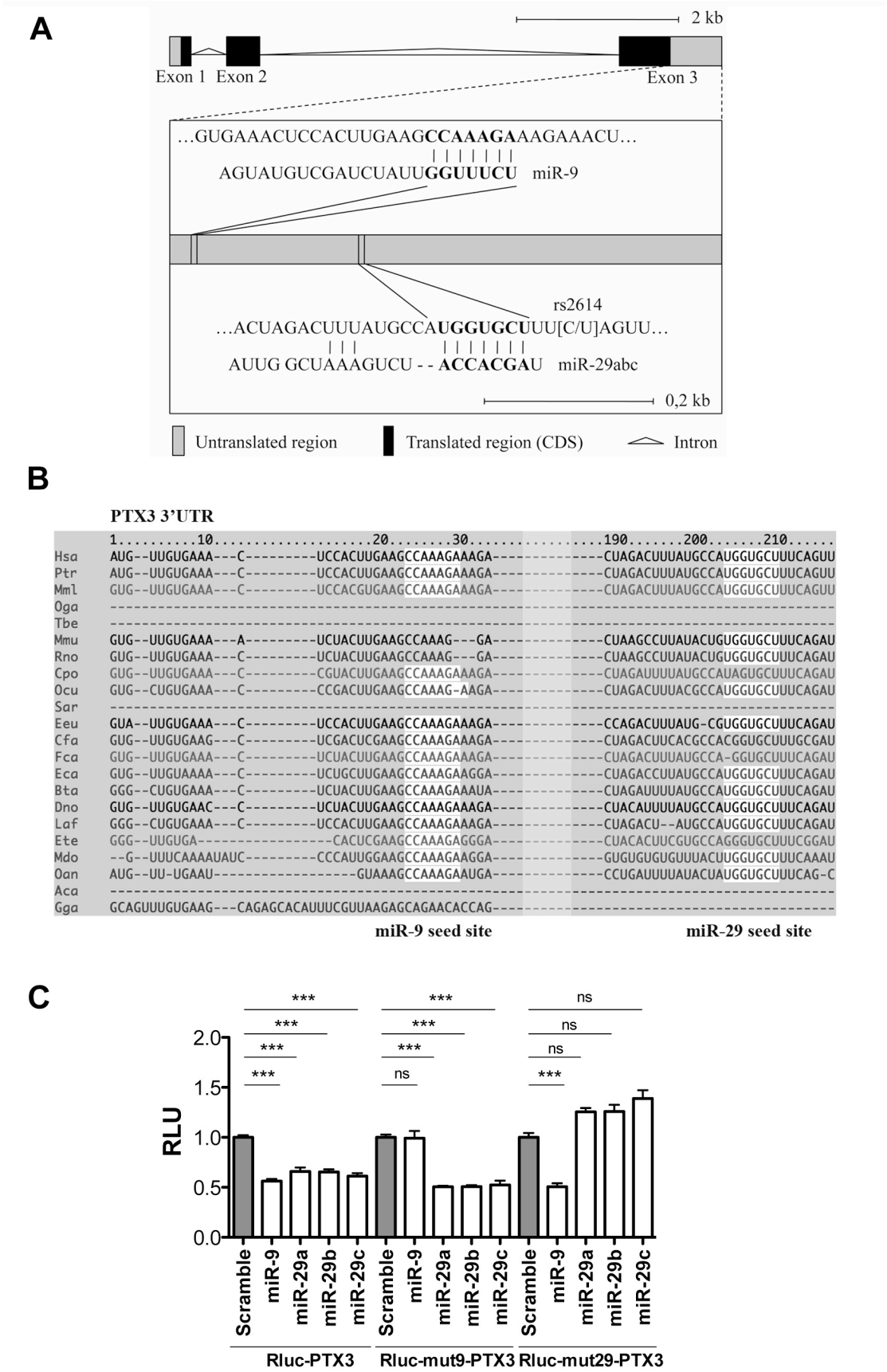

Figure 6 | **PTX3 is a direct target of miR-9 and miR-29.** (A) *PTX3* gene has been mapped on chromosome 3 (3q25.3) and it is composed of three exons. Its 3' UTR is 608 bp-long and miR-9 and miR-29 seed regions are located 24 and 204 bp downstream the PTX3 mRNA stop codon, respectively.

The rs2614 SNP is located three bp downstream miR-29 target site. (B) miR-9 and miR-29 seed regions on PTX3 3' UTR are evolutionary conserved, as shown by TargetScan, in primates and mammals in general. (C) HEK293T cells were co-transfected with 100 ng of the indicated luciferase constructs (Rluc-vectors) together with 100 nM pre-miR-9, pre-miR-29, or the scramble control (grey columns). Results are expressed as mean (± SEM, n=3) of the ratio between renilla luciferase and firefly control luciferase activities (RLU) adjusted to 1. ***: $p < 0.001$; ns: $p > 0.05$.

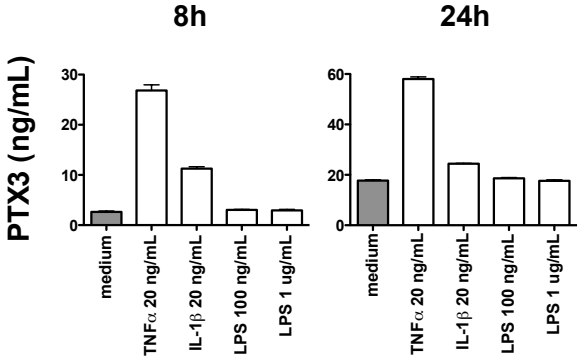

Figure 7 | **PTX3 production by 8387 cells.** Results are represented as mean (± SEM, n=3) of PTX3 released (ng/mL) by 8387 cells without any stimuli (grey column) or treated with TNFα (20 ng/mL), IL-1β (20 ng/mL) or LPS (100 ng/mL and 1μg/mL).

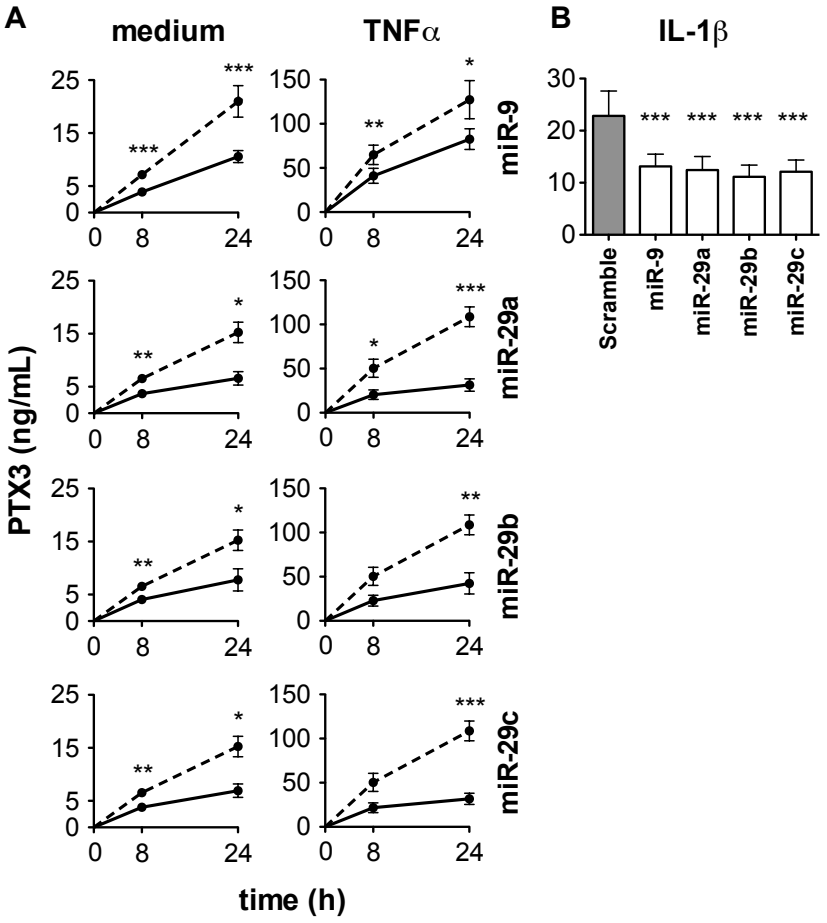

Figure 8 | **Effect of miR-9 and miR-29 on PTX3 production.** 8387 cells were transfected with 100 nM pre-miR-9, pre-miR-29abc or scramble control. PTX3 production (ng/mL) was measured by home-made ELISA and results are represented as mean (± SEM, n=6 for miR-9 and n=3 for miR-

29abc). (A) Each graph shows the time course of PTX3 production for the considered miR (−•−) and for the scramble control (--•--). Graphs on the left are referred to the basal protein production, graphs on the right to the TNFα-induced (20 ng/mL) PTX3 production. (B) PTX3 production after 8h of IL-1β stimulation (20 ng/mL) is represented in the bar graph. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Inflammation-related microRNAs affect PTX3 production via transcriptional regulation

Mononuclear phagocytes are a major source of PTX3 during inflammatory responses[50,297]. At the same time, proinflammatory stimuli induce a distinct set of microRNAs in human mononuclear cells, including miR-9, miR-132, miR-146a, miR-155, miR-187, and the miR-99b~7e~125a cluster[210,288]. Therefore, we investigated the potential role of these inflammation-related microRNAs on PTX3 regulation in luciferase reporter assay and on its production in 8387 cells transfected with mimics. PTX3 basal production was significantly impaired by miR-132. miR-146a and miR-155 significantly down-regulated PTX3 production in IL-1β and TNFα stimulated cells (Fig. 9). The miR-99b~7e~125a cluster and miR-187 did not affect PTX3 production at any condition tested (Fig. 10). Consistent with the lack of corresponding seed regions in the PTX3 3' UTR, apart from miR-9, none of the other miRNAs tested was able to affect PTX3 3' UTR stability in luciferase reporter assay (data not shown).

Since our results indicated that miR-132, miR-146a and miR-155 down-regulated PTX3 production without directly targeting PTX3 mRNA, we hypothesized they might have an impact on PTX3 transcription. To test this hypothesis, the *PTX3* promoter sequence (gi:1679604) was cloned in the PGV-B2 reporter vector and transiently co-transfected with different miRNA precursors in HEK293T cells. Consistent with our hypothesis, miR-146a and miR-155 significantly limited the *PTX3* promoter activity induced by TNFα stimulation, while miR-132 was ineffective (Fig. 11). This experiment also highlighted that miR-9 did not interfere with the promoter activity, whereas miR-29abc impaired *PTX3* promoter activity. This reveals two levels of control exerted by miR-29 family members on PTX3 production (Fig. 11).

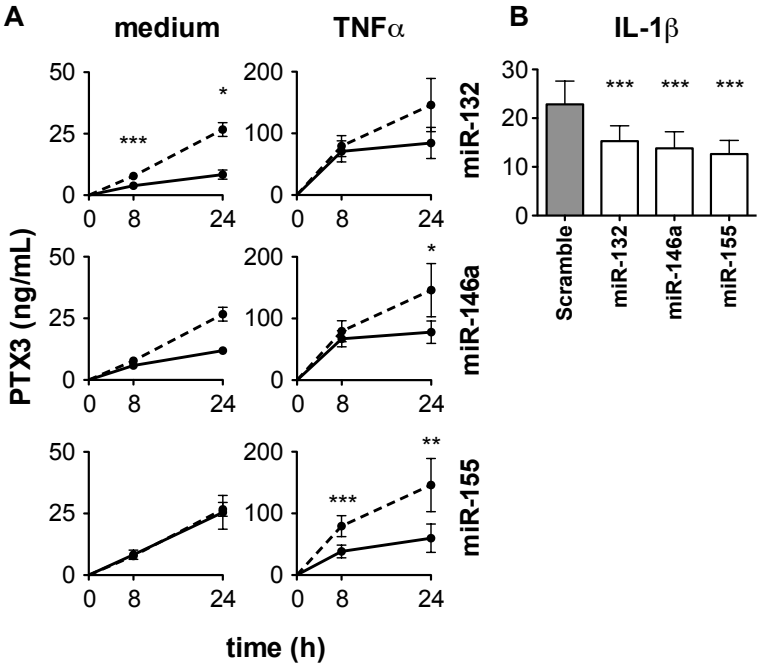

Figure 9 | **Effect of miR-132, miR-146a and miR-155 on PTX3 production.** 8387 cells were transfected with 100 nM pre-miR-132, pre-miR-146a, pre-miR-155 or scramble control. PTX3 production (ng/mL) is expressed as mean (± SEM, n=3). (A) In each graph the time course of PTX3 production is represented for the considered miR (−•−) and for the scramble control (--•--) in basal condition (left column) and after TNFα stimulation (20 ng/mL, right column). (B) The bar graph displays the PTX3 production after 8h of IL-1β stimulation (20 ng/mL). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

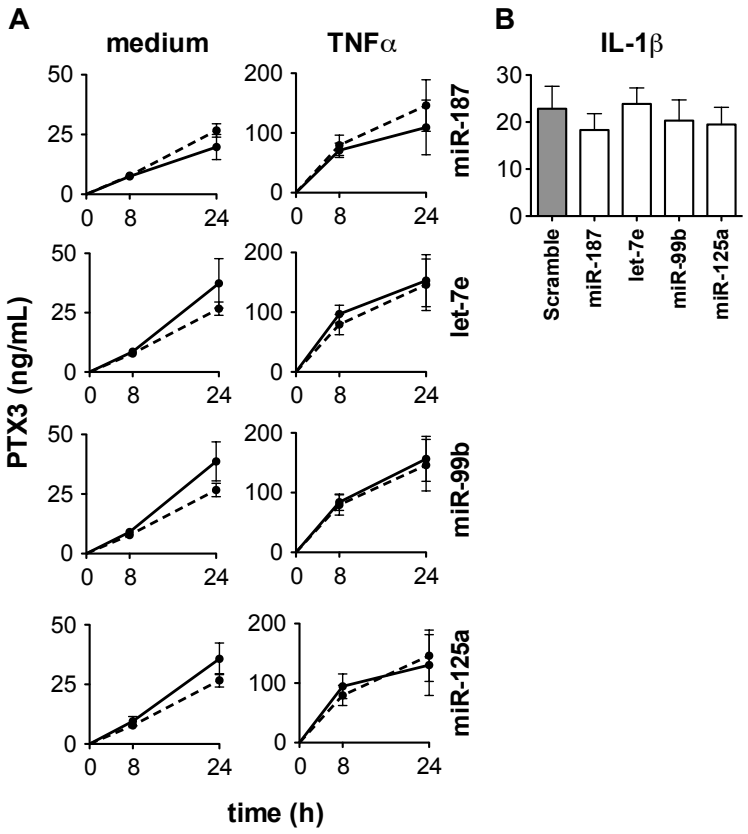

Figure 10 | **Effect of miR-187 and miR-125a-let-7e-miR-99b cluster on PTX3 production.** 8387 cells were transfected with 100 nM pre-miR-187, pre-let-7e, pre-miR-99b, pre-miR125a or scramble control. PTX3 production (ng/mL) is represented as mean (± SEM, n=3). (A) Each graph shows the time course of PTX3 production for the considered miR (−•−) and for the scramble control (--•--).Graphs on the left are referred to the basal protein production, graphs on the right to the TNFα-induced (20 ng/mL) PTX3 production. (B) PTX3 production after 8h of IL-1β stimulation (20 ng/mL) is represented in the bar graph.

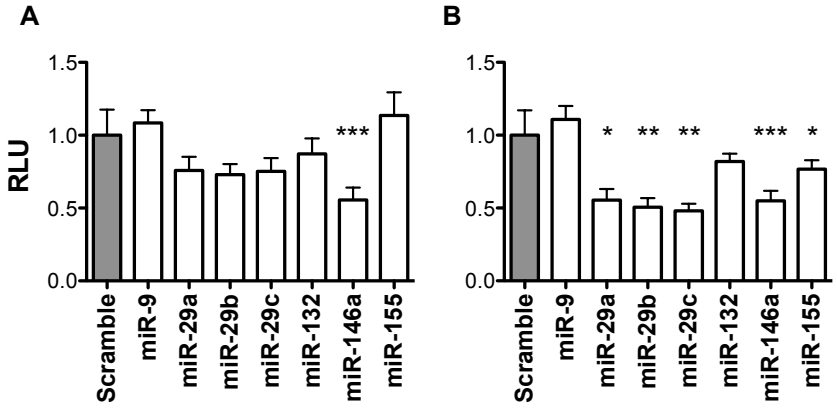

Figure 11 | **Effect of miR-9, miR-29, miR-132, miR-146a and miR-155 on PTX3 transcription.** 50 ng PGV-B2 vector expressing whole *PTX3* promoter sequence fused to the firefly luciferase CDS has been co-transfected in HEK293T cells together with 50 ng pRL-TK vector expressing renilla luciferase as normalizer and with 100 nM pre-miR-9, pre-miR-29, pre-miR-132, pre-miR-146a, pre-miR-155 and scramble control. The experiment has been conducted in basal condition (A) and upon TNFα (20 ng/mL) stimulation (B). Results are expressed as mean (± SEM, n = 3) of the ratio between firefly luciferase and renilla luciferase control activities (RLU) adjusted to 1. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

miR-181c affects PTX3 production acting at two levels of control

PTX3 is a complex multimeric protein, whose function requires the correct formation of intra- and inter-chain disulphide bonds[60,62,96]. For PTX3 this function is fulfilled by ERp18[298], a member of the protein disulphide isomerase (PDI) oxidoreductases responsible for catalysing formation, isomerisation and reduction of disulphide bridges in the endoplasmic reticulum[299].

Hence, we investigated the possibility of a third level of microRNA-dependent regulation of PTX3 production exerted as post-translational regulation via direct targeting of ERp18. *In silico* analysis predicted multiple seeds for miR-181 family members in the ERp18 3' UTR (Fig. 12A). A luciferase reporter assay in HEK293T cells was performed to verify the direct interaction of the four miR-181 family members with the ERp18 3' UTR. As shown in Fig. 12B, miR-181 strongly impaired Rluc-Erp18 luciferase activity as compared to the scramble control, reducing the luciferase expression by approximately 55%. When miR-181 8-mer seed region was mutated on ERp18 3' UTR, the luciferase expression was not completely restored. However, it was sufficient that the 7–mer long seed site was mutated in combination with the 8-mer seed region, for restoring the luciferase activity. Collectively, these data suggest that the 8-mer and 7-mer seed regions in ERp18 3' UTR are specific sites for the interaction miR-181:ERp18 mRNA.

As miR-181c has been previously related to the inflammatory response, being up-regulated by LPS in mouse primary macrophages[300], we focused our attention on this specific member of the miR-181 family. To validate the role of miR-181c on PTX3 production, 8387 cells were transfected with 100 nM pre-miR-181c and stimulated or not with TNFα or IL-1β. miR-181c significantly down-regulated the basal PTX3 production as well as the protein production induced by TNFα (Fig. 13A) or IL-1β (Fig. 13B). To verify whether PTX3 production was impaired by miR-181c through its activity on ERp18 mRNA, ERp18 was silenced by ERp18 siRNA. ERp18 mRNA silencing was validated by real-time PCR (data not shown), and Western blot analysis showed a comparable effect of ERp18 siRNA and miR-181c in down-regulating ERp18 (82.8% and 72.8% inhibition, respectively; Fig. 13C).

The effects of pre-miR-181c and ERp18 siRNA transfection on basal and TNFα-induced PTX3 production by 8387 cells were then compared (Fig. 14). 24h after transfection basal PTX3 production was significantly down-regulated by miR-181c

(48%) and only marginally by siR-ERp18 (13%). However, the combination of both the interfering RNAs caused a 60% inhibition in basal condition ($p<0.01$) and a 52% inhibition in TNFα-induced PTX3 production ($p<0.05$). When miR-181c and siR-ERp18 were transfected independently, TNFα-induced PTX3 production was less important (45 and 29%, respectively). At an early time point, the basal PTX3 production was neither affected by miR-181c, by siR-ERp18 nor by their combination.

Hence, miR-181c targeted ERp18 mRNA leading to an inhibition of ERp18 protein expression similar to that obtained by the silencing RNA directed against ERp18. Nevertheless, this strong activity was not sufficient to explain the down-regulation of PTX3 production exerted by mir-181c. Actually, siR-ERp18 did not cause the same down-regulation of PTX3 obtained with miR-181c. Moreover, miR-181c and siR-ERp18 exerted an additive effect on PTX3 production, suggesting that miR-181c had other targets, than ERp18, involved in PTX3 expression. Hence, we tested the activity of miR-181c on the signalling pathway responsible for *PTX3* promoter activation in HEK293T cells. The analysis revealed a miR-181c-dependent inhibition of TNFα-induced *PTX3* promoter-regulated luciferase expression (Fig. 15).

Taken together, these results show the ability of miR-181c to regulate PTX3 expression at two different levels. In fact, it down-modulates the signalling cascade responsible for *PTX3* transcription and it abolishes the production of ERp18, involved in PTX3 folding.

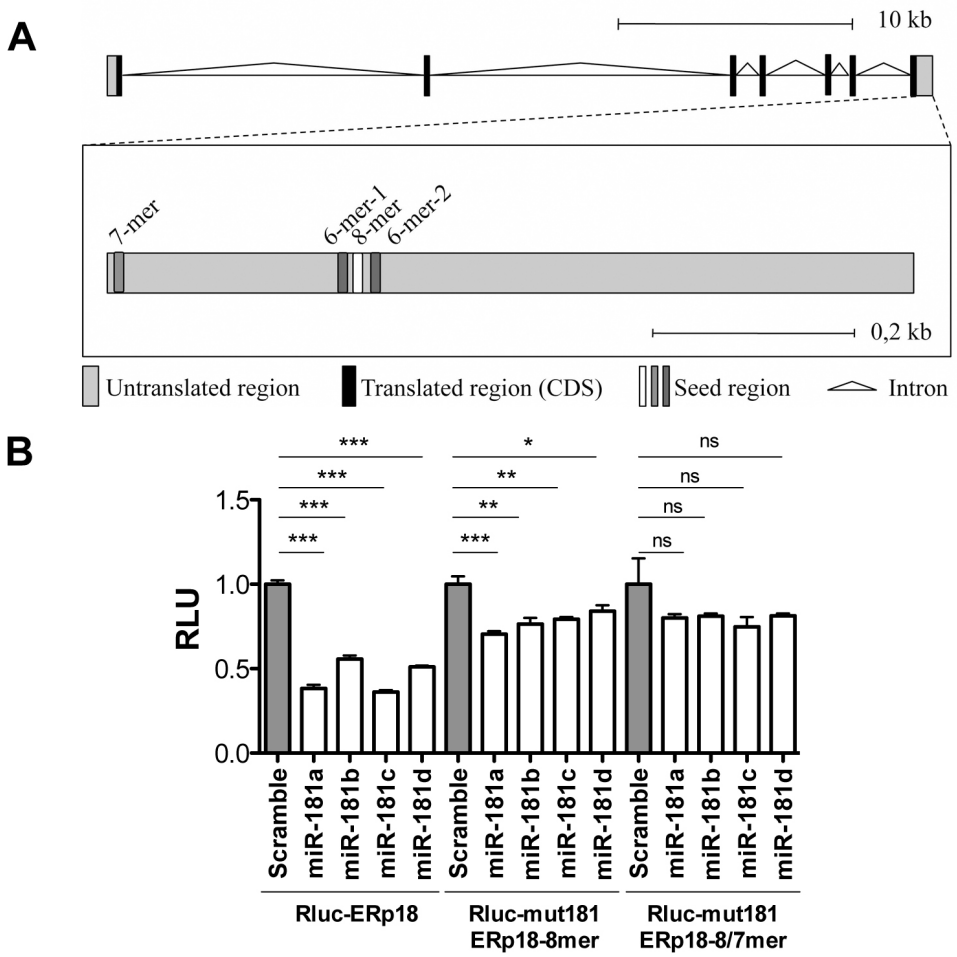

Figure 12 | Schematic representation of ERp18 3' UTR and effect of miR-181 family members on it. (A) Schematic representation of the four miR-181 target sites on the ERp18 3' UTR: one 8-mer long and one 7-mer long seed regions are located 300 bp and 25 bp downstream the stop codon, respectively. Two additional 6-mer long seed sites are also present in the ERp18 3' UTR. (B) HEK293T cells were co-transfected with 100 ng of the indicated luciferase constructs (Rluc-vectors) together with 100 nM pre-miR-181 or scramble control (grey columns). Results are expressed as mean (± SEM, n=3) of the ratio between renilla luciferase and firefly control luciferase activities (RLU) adjusted to 1. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; ns: $p > 0.05$.

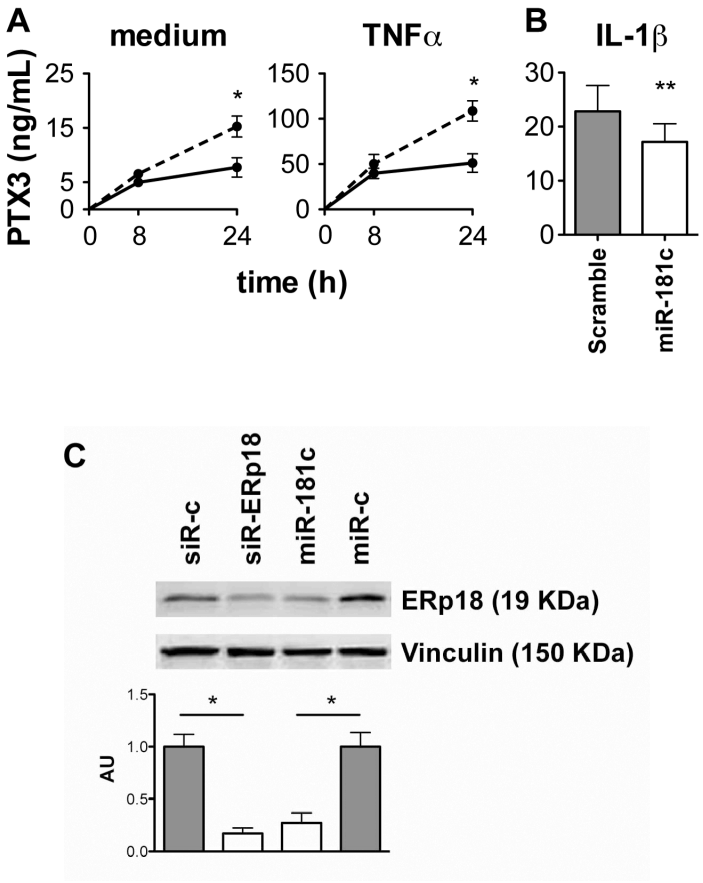

Figure 13 | **Effect of miR-181c on PTX3 production.** (A and B) 8387 cells were transfected with 100 nM pre-miR-181c (−•−) or scramble control (--•--) and PTX3 production (ng/mL) in basal condition and after TNFα (20 ng/mL, panel A) or IL-1β (20 ng/mL, panel B) stimulation was measured. Results are presented as mean (± SEM, n=3). (C) 8387 cells transfected with siR-ERp18, miR-181c and respective controls (siR-c and miR-c) were lysed and extracted proteins were loaded on SDS-PAGE gel. Nitrocellulose filter was hybridised with pAb-anti-ERp18 or pAb-anti-vinculin as normalizer. A Western Blot image of 1 experiment (representative of 2) is reported together with a bar graph derived from a densitometric analysis (n=2). **: $p < 0.01$; *: $p < 0.05$.

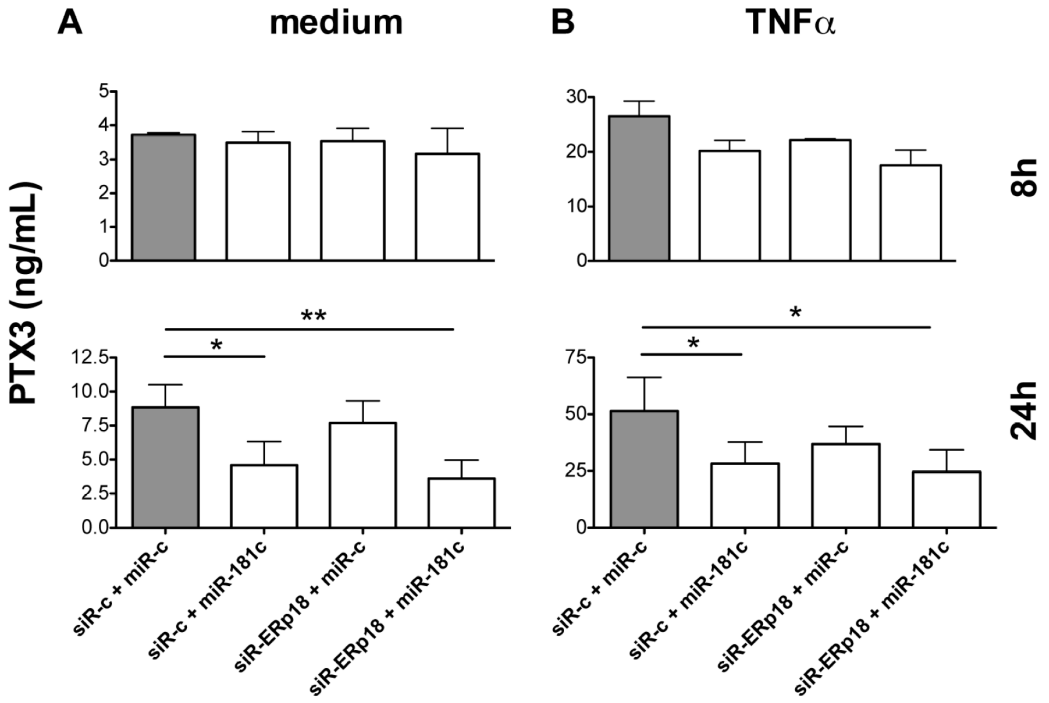

Figure 14 | **Effect of miR-181c and siR-ERp18 co-transfection on PTX3 production.** 8387 cells were co-transfected with combinations of the indicated small interfering RNAs (50 nM each). PTX3 production (ng/mL) was evaluated in basal condition (panel A) and after TNFα stimulation (20 ng/mL, panel B). Results are expressed as mean (± SEM, n=2). **: $p < 0.01$; *: $p < 0.05$.

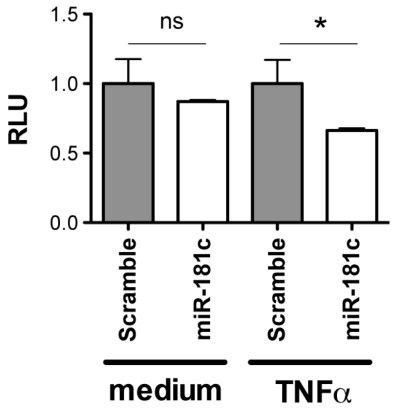

Figure 15 | **miR-181c activity on *PTX3* promoter.** 50 ng of PGV-B2 vector expressing the whole *PTX3* promoter sequence fused to the firefly luciferase CDS has been co-transfected in HEK293T cells together with 50 ng pRL-TK vector expressing renilla luciferase as normalizer and with 100 nM pre-miR-181c or scramble control. The experiment has been conducted in basal condition and upon TNFα (20 ng/mL) or IL-1β stimulation (20 ng/mL). Results are expressed as mean (± SEM, n = 3) of the ratio between firefly luciferase and renilla luciferase control activities (RLU) adjusted to 1. *: $p < 0.05$; ns: $p > 0.05$.

Discussion

PTX3 has been the first long pentraxin to be described in the 1990s and in twenty years it has been well characterized from several points of view. Different signalling cascades lead to PTX3 production, depending on the cell type and on the stimuli. *PTX3* promoter contains numerous transcription factor binding sites including Pu1, AP-1, NF-κB, SP1 and NF-IL-6 sites. In fibroblasts, AP-1 is involved in the basal transcription of PTX3, whereas the NF-κB-binding site is fundamental for the transcriptional response to TNFα and IL-1β, even if also Sp1 site seems to be important for a complete TNFα and IL-1β response[49]. In alveolar epithelial cells TNFα-induced PTX3 production is mediated by the JNK pathway[57]. The activation of both NF-κB and AP-1 transcription factors is important for the up-regulation of PTX3 expression in human aortic endothelial cells stimulated with the acute phase protein SAA[301]. In a model of acute myocardial ischemia and reperfusion in mice, the PTX3 mRNA induction has been linked to the IL-1R-MyD88 pathway[56].

In silico analysis predicted and *in vitro* experiments confirmed that miR-9 and miR-29 control PTX3 mRNA stability directly acting on its 3' UTR. Whereas miR-9 activity is dependent on its interaction with PTX3 mRNA, miR-29 controls TNFα-induced PTX3 expression also through the modulation of the signalling cascade that culminates in the *PTX3* promoter activation. This last ability of miR-29 is shared with other inflammation-related microRNAs induced by pro-inflammatory stimuli in monocytes, such as miR-132, miR-146a and miR-155. Whereas miR-132 negatively controls both basal and induced PTX3 production, miR-146a and miR-155 predominant activities are exerted after TNFα and IL-1β-stimulation. These observations suggest that miR-132, miR-146a and miR-155 indirectly affect PTX3 production acting on unidentified regulators of PTX3 biosynthesis. miR-181c data, reported here, show how a microRNA-mediated negative regulation on a single molecule can be very complex and exerted at several levels. Actually, miR-181c operates a down-regulation of PTX3 expression impairing *PTX3* promoter activity and interfering with the correct folding of PTX3.

miR-9 has an essential role in mammalian brain development[302,303]. miR-9 also targets the key metastasis-suppressing protein E-cadherin leading to increased cell motility and invasiveness in cancer metastasis[304]. Only in 2008 for the first time miR-9 has been demonstrated to be involved in the inflammatory response. miR-9

transcription is induced in monocytes and PMN by LPS, TNFα and IL-1β and it is involved in maintaining constant the levels of the NF-κB transcription factor subunit NFKB1/p105, avoiding an exacerbated immune response[288]. In our experiments miR-9 does not affect *PTX3* promoter activity neither in basal conditions nor after TNFα stimulation, suggesting a NF-κB-independent PTX3 production in this experimental condition.

miR-132 has been first linked to neuronal morphogenesis in 2005[305] and, only one year later, it has been associated to the inflammatory response[264]. Interestingly, miR-132 regulates innate antiviral immunity by targeting the p300 transcriptional co-activator[286]. Actually, p300 has been described as a complex protein with several functional domains. On one hand, it acts as an adaptor molecule, interacting with transcription factors and modulating the expression of target genes transcribed by all three RNA polymerases. On the other hand, behaving as a histone acetyltransferase, p300 alters the local chromatin structure, allowing transcription. In our experiments, miR-132 overexpression resulted in an impaired PTX3 production, whereas any effect of miR-132 was reported on *PTX3* promoter activity. Hence, we hypothesize a global impairment of transcription due to miR-132-dependent p300 down-regulation, exerted on both the reporter gene (firefly luciferase) and the house-keeping gene (renilla luciferase). This could explain the lack of activity detected on *PTX3* promoter, but could justify the impairment of PTX3 production observed after miR-132 overexpression.

In 2006, Baltimore *et al.* demonstrated the capacity of miR-146 to negatively regulate the immune response. Actually, after its transcriptional induction by LPS, miR-146 controls through a feedback mechanism the TLR signalling, directly interacting with TRAF6 and IRAK1[264]. Actually, TRAF6 participates in the IL-1R/TLR signalling and also in the TNF-induced cascade. The activation of both these pathways can lead to the regulation of AP-1 and NF-κB transcription factors[306]. Also IRAK1 has been proposed as a molecule that integrates LPS, TNFα and IL-1β signalling pathways[307]. Hence, TRAF6 and IRAK1 miR-146-mediated targeting could be responsible for the *PTX3* promoter modulation and for the protein down-regulation seen in our experiments.

miR-155 has been the first oncogenic miRNA to be discovered and its high expression has been associated with B-cell and Hodgkin's lymphomas[308]. miR-155

knockout mouse was the first generated miRNA-deficient animal and it elucidated the main importance of miR-155 in driving the immune response[283]. The TLR/IL-1 inflammatory pathway has been identified as a general target of miR-155[275] that interferes also with the TNFR1-mediated signalling[309]. These abilities of miR-155 could explain the down-regulation of PTX3 production exerted after TNFα and IL-1β stimulation.

In human monocytes, the concomitant transcriptional induction by pro-inflammatory stimuli of PTX3 and miR-9, miR-132, miR-146a and miR-155 could be a mechanism by which immune cells keep under control the levels of PTX3 and avoid an excessive immune response.

The first description of miR-29 dates back to 2005 when Smirnova and colleagues reported a differential expression of miR-29 in different cell types of the central nervous system[310]. One year later, miR-29 and miR-181 have been associated to B cell chronic lymphocytic leukaemia (B-CLL). In these cells, both families are down-regulated and *TCL1* oncogene, a direct target of these microRNAs, is consequently up-regulated, facilitating the establishment of a B-CLL aggressive form[311]. Moreover, miR-29 has been associated with the fibrosis of different organs[312,313]. Interestingly, the group of Olson highlighted the role of miR-29 in cardiac fibrosis after myocardial infarction. In this context, miR-29 is preferentially expressed by the fibroblast population[314]. miR-29 is also involved in the post-transcriptional control of proteins of the extracellular matrix[315], where PTX3 is expressed[1]. In our hands, miR-29 exerts a direct action on PTX3 mRNA, but it also shows an indirect effect on molecules belonging to the PTX3-inducing signalling pathway. Actually, miR-29 is regulated and in turn it regulates the Wnt signalling, positively modulating osteoblast differentiation[316,317]. p85α and CDC42 are also targets of this miRNA[318]. p85a is the regulatory subunit of PI3K, a kinase already shown to be fundamental in HDL-dependent PTX3 production through the lysosphingolipids receptors-PI3K/Akt axis activation in endothelial cells[58]. CDC42 belongs to the Rho family GTPases and is known to positively modulate several transcription factors, among which AP-1[319-321]. All these pathways are well characterized in inflammatory response and can potentially regulate PTX3 production, since miR-29 modulates *PTX3* promoter activity.

Previously described as a hematopoietic-specific micro-RNA in mouse, miR-181 has been also detected in normal human B cells, T cells, monocytes and granulocytes[322]. Recently, Luers and colleagues analysed the miRNA profile of human primary macrophages differentiated *in vitro* from peripheral blood $CD14^+$ monocytes. miR-181ab have been consistently detected in these cells together with miR-29a, miR-132, miR-146a, miR-155 and miR-99b~7e~125a cluster[323]. In the same year, Schwind *et al.* provided the first evidence that the expression of miR-181a is associated with the clinical outcome of patients with cytogenetically normal acute myeloid leukemia (CN-AML)[324]. Later, the down-regulation of miR-181ab has been correlated to activated B cells and CLL cells[325]. Among the four members of miR-181 family, miR-181c is positively regulated by Akt1 in LPS-activated primary murine peritoneal macrophages[300] where a possible interaction between miR-181 and PTX3 can be supposed. The highlighted capacity of miR-181c to modulate PTX3 expression amplifies the spectrum of PTX3-regulating miRNAs.

In summary, the results reported in this study emphasize the well-known capacity of microRNAs to play the role of fine-tuners of a specific signalling cascade (Fig. 16). Basal and TNFα/IL-1β-induced PTX3 expression are regulated by miR-9 and miR-29 at the post-transcriptional level. *PTX3* promoter activation can be modulated by miR-146a, in both basal and TNFα stimulated conditions, and by miR-29, miR-155 and miR-181c after TNFα stimulation. A further level of control is exerted by miR-181c through its negative regulation on ERp18 mRNA, interfering presumably with the correct folding of PTX3. Since PTX3 is produced by several cell types, future experiments are required to validate our results in different cellular contexts: monocytes, macrophages and fibroblasts, but also DCs, SMC and EC. It will be also important to investigate the PTX3-regulating microRNAs network *in vivo*.

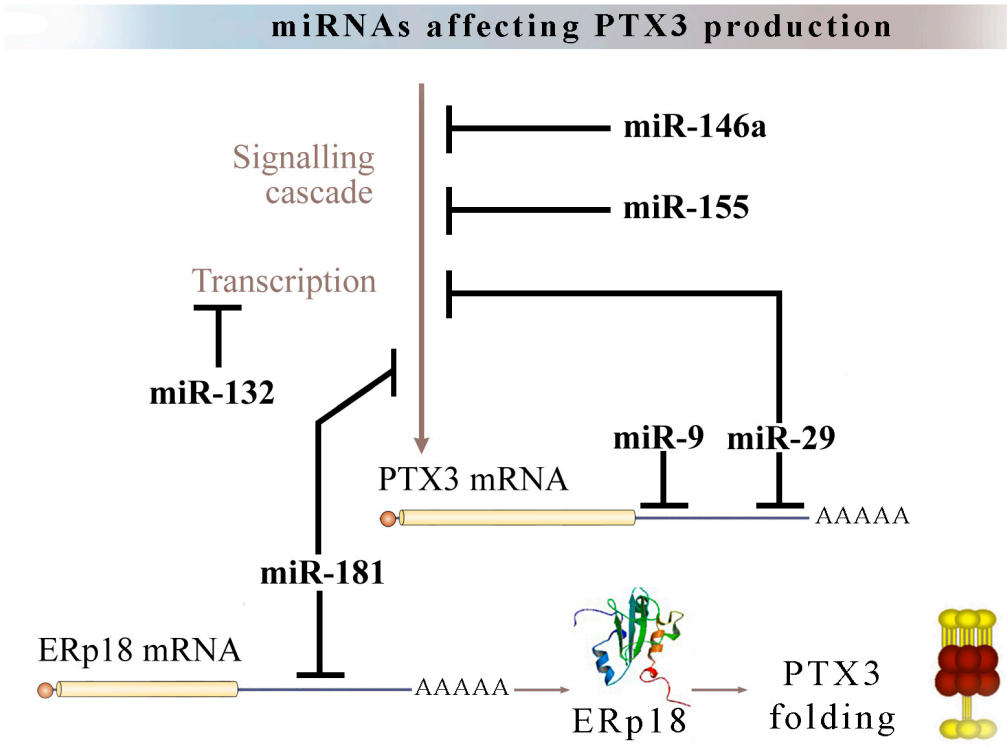

Figure 16 | The complex micro-RNA mediated regulation of PTX3 expression.

Concluding remarks

The work described in this thesis reveals that PTX3 expression can be regulated at two different levels by *PTX3* SNPs, on one hand, and microRNAs, on the other hand.

Data from the first work of this thesis suggest the possibility to distinguish, at least in the Caucasian population, two main groups of subjects according to their *PTX3* genotypes and, consequently, to their PTX3 basal levels in the blood. We hypothesize that these differences could have an impact in the contexts in which PTX3 activity is involved. Indeed, the PTX3 haplotypes studied here have been associated to innate resistance to selected pathogens, inflammation and female fertility. However, this study indicates that even if *PTX3* genetic variants affect PTX3 plasma levels both in healthy subjects and AMI (acute myocardial infarction) patients, these variants do not correlate with a different susceptibility to AMI.

The last work presented here shows for the first time that PTX3 expression can be regulated by a plethora of microRNAs at different levels. Some of them act directly on PTX3 mRNA, such as miR-9 and miR-29. In parallel, miR-146a, miR-155, miR-29 and miR-181c control molecules of the signalling pathway for *PTX3* transcription. Finally, miR-181c down-regulates the expression of ERp18, a molecule involved in the correct folding of PTX3.

Since miRNAs are potential molecular targets of therapeutic approaches, it is essential to understand their functions and targets in human cells. Our data report a fundamental implication of microRNAs in the control of PTX3 production. Starting from these data, further efforts are needed to elucidate in which cell type each interaction occurs and the consequences of a disruption of the microRNA complex network in terms of PTX3 production and biological effects.

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