# Modulation of genes involved in inflammation and cell death in atherosclerosis-susceptible mice

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# Chapter 7

# General Discussion

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# Discussion

In this thesis we focussed on atherosclerosis as the primary cause of cardiovascular disease. Atherosclerosis is a chronic disease, characterised by inflammation and the focal accumulation of lipid laden foam cells covered by a fibrous smooth muscle cell rich cap[1]. Progression of a lesion depends on the balance of pro-atherogenic and anti-atherogenic factors. These factors determine lesion composition and thereby plaque stability and vulnerability to rupture. Since accumulating evidence suggests that inflammation and cell death are important processes in the onset and progression of atherosclerosis, we investigated the role of several genes and gene products involved in (both) inflammation and cell death in the vessel wall and their effect on atherosclerosis. To this end, we used several strategies to modulate gene expression in different atherosclerosis-susceptible mouse models.

### *Cell death and Atherosclerosis*

Cell death is a feature of atherosclerosis and can occur in every stage of disease development[2]. Both apoptotic and necrotic cell death are seen in atherosclerotic plaques. Abnormal occurrence of apoptosis can take place in the plaque, including attenuation or acceleration of apoptotic cell death[3]. By counterbalancing proliferation, apoptosis may limit cell build-up in the intimal compartment. In the initial stage of atherosclerosis, elimination of lipid laden foam cells may even lead to regression. In contrast, attenuation of apoptosis, possibly as a result from the presence of several anti-apoptotic factors[4;5], may increase tissue cellularity and promote plaque progression. Necrosis and the necrotic core are features of the advanced atherosclerotic plaque. Inflammatory cytokines, proteases, radicals and coagulation factors derived from in and around the necrotic core are generally thought to promote atherosclerosis[6-9]. Apoptotic and necrotic cell death may contribute to the development of two major pathological characteristics of advanced atherosclerotic lesions. First, the centre of the plaque contains many dead cells or cell debris. Although plaques bear markers of apoptosis, necrotic cell death may also occur in the lipid core area, hence, the term necrotic lipid core[10]. Second, apoptosis causes SMC loss as atherosclerosis progresses, yielding a lesion with a dense extra-cellular matrix with a sparse cell population, termed a hypocellular fibrotic lesion.

In this thesis we studied whether several genes involved in cell death play a role in atherosclerosis. Our study on the major apoptotic cell death inducer FasL in plaque vulnerability showed that FasL-induced increased apoptosis did not result in significant differences in cellularity and in total plaque area between treated and control groups **(chapter 3)**. In a similar study, ectopic p53-induced apoptosis neither caused a difference in total lesion area[11]. Furthermore, this thesis showed that increased cell death in the form

of necrosis is not necessarily linked to an increase in atherosclerosis. Whole body deletion of TNFα did not result in a difference in atherosclerotic lesion size, while apoptosis was decreased and necrosis was increased **(chapter 2)**. This was corroborated in a study on the specific role of macrophage p53 in atherosclerosis (Boesten and Zadelaar *et al.*, submitted), in which no difference in total plaque area was found upon p53 deficiency, despite an increase in necrotic area. In conclusion, and in contrast to the general beliefs on apoptosis and necrosis, apoptosis is not always linked to reduced tissue volume and necrosis not to increased tissue volume. However, the abovementioned studies do show that interfering with apoptotic or necrotic cell death can induce a change in composition, and thereby a more vulnerable phenotype, without affecting tissue volume.

The fact that apoptotic cells in the atherosclerotic plaque accumulate, suggests that the system for scavenging dead cells operates poorly. Some apoptotic cells or bodies remain mummified rather than undergo removal by phagocytosis[10]. This can be caused by a number of mechanisms: 1. intracellular lipid accumulation in macrophages and SMCs in the plaque may attenuate the ability to engulf and digest apoptotic cells or bodies, 2. increased apoptosis of macrophages may decrease phagocytotic clearance, 3. cross linking of macromolecules, such as proteins, nucleic acids and carbohydrates may stabilize apoptotic cells in the tissue, and 4. the presence of increased amounts of modified lipoproteins and other ligands for scavenger receptors may competitively block receptor-mediated phagocytosis of apoptotic cells by macrophages[3]. As yet, what happens with residing apoptotic cells remains subject to speculation. One possibility is that secondary necrosis is induced in residing apoptotic cells near the necrotic core due to the present hypoxia, excess intracellular lipid, depletion of nutrients, and other factors[12]. These cells may contribute to enlargement of the necrotic core. This theory was previously supported by a study in which inhibition of p53 in mouse embryonic fibroblasts results in a shift of NO-induced cell death towards relatively more necrosis and less apoptosis[13;14]. In our studies TNFα **(chapter 2)** and macrophage p53 deficiency (Boesten and Zadelaar *et al.*, submitted) induced a shift from apoptosis to necrosis, indicated by a decreased number of apoptotic cells and an increased necrotic core area. From these findings we can derive that (macrophage) apoptosis is beneficial to the plaque. However, when apoptosis can not be executed properly, due to the unfavourable environment, necrosis may follow. The alternative necrotic death pathway is generally considered to be more detrimental, since necrosis leads to the release of pro-inflammatory and pro-thrombotic substances.

***Inflammation and Atherosclerosis***

Several genes and gene products in this thesis were studied, which showed to have a direct or indirect effect on inflammation in atherosclerotic plaque development. Macrophages

have a prominent role in the inflammatory state of the atherosclerotic plaque. They produce cytokines and chemokines that attract additional macrophages, T-cells and other immunomodulatory cells to the plaque[6]. These cells may produce a plethora of additional cytokines. One of the most important inflammatory cytokines is TNFα[15]. The result that deletion of TNFα showed no effect on atherosclerotic lesion size was therefore surprising. However, analysis of lesion composition did show a shift from lesion apoptosis to necrosis **(chapter 2)**. The alternative necrotic death pathway may be slower, but all the more detrimental, since necrosis leads to the release of pro-inflammatory and pro-thrombotic substances. It might be this enhanced inflammatory environment that stimulated the progression of the plaque to a more advanced phenotype. A study on the specific role of macrophage p53 deficiency showed a similar shift to necrosis, indicating a more inflammatory environment (Boesten and Zadelaar *et al.*, submitted). On inflammatory macrophages and T-cells the cell death inducer FasL is present[16]. At first sight, FasL may seem anti-inflammatory, being present at immunoprivileged sites, preventing entrance of inflammatory cells[17]. At the surface of ECs FasL may serve a similar function in inhibiting leukocyte extravasation[18]. In contrast, ectopic expression of FasL can result in tissue damage and induce inflammatory responses characterized by monocyte and neutrophil infiltration[19]. This observation is supported by our study, in which ectopic overexpression of FasL in the caps of pre-existing atherosclerotic plaques also resulted in increased monocyte adhesion **(chapter 3)**. It is suggested that the Fas death machinery switches on a set of pro-inflammatory genes such as chemoattractant MCP-1 and IL-8[20] and thereby induces the recruitment of additional monocytes. Collectively, in addition to their function in cell death, in this thesis TNFα, FasL and p53 contribute to the inflammatory state of the atherosclerotic plaque, likely via the affection of macrophages.

The dual PPARα/γ agonist tesaglitazar showed atherosclerosis reducing capacities beyond its cholesterol lowering effect. These “pleiotropic” effects are suggested to be associated with anti-inflammatory effects **(chapter 6)**. Anti-inflammatory effects were evident from lowering total lesion area and inflammatory macrophage content. Adhering macrophages were also reduced. Furthermore NF-κB was measured as a central mediator of inflammation. The presence of NF-κB and its active form was found to reflect plaque size and severity. Next to inflammation, NF-κB is also a mediator of apoptosis, proliferation and differentiation. In atherosclerosis NF-κB can be involved in all stages of development. While only measured in **chapter 6**, the genes and proteins described in all chapters could have this mediator in common and can influence or be influenced by NF-κB[21;22] (Figure 2 introduction). Up to now only indirect studies on NF-κB regulators were performed. Since NF-κB is such a central player in immunity, direct affection would have

detrimental effects. These studies showed different outcomes, ranging from increasing, decreasing, and no effect on atherosclerosis (for overview see review[21]). Future studies with conditional models should show more unequivocal results and will further clarify the central inflammatory role of NF-κB in atheroslerosis. The results from **chapter 6** may take part in the accumulation of evidence to suggest that the beneficial effect on atherosclerosis of PPARα/γ agonists may be associated with their effect on NF-κB and thereby cell death and inflammation.

***Cell Death and Inflammation in Atherosclerotic Plaque Vulnerability***

In the previous paragraphs cell death and inflammation were discussed in the context of atherogenesis and plaque progression. However, these processes might be the underlying mechanism to the development of vulnerable plaques, possibly leading to the critical end stages of plaque rupture. Cell death can contribute to plaque instability. EC death can initiate plaque formation or later on trigger thrombotic events[23]. SMC death can cause destabilization of the cap for numerous reasons[24]. Overexpression of FasL in the cap of pre-existing lesions resulted in increased apoptosis one day after incubation. Although no difference in total lesion area was observed, apoptosis stimulated a transition towards a vulnerable plaque phenotype as analyzed 14 days after incubation. This was demonstrated by endothelial discontinuity, intra plaque hemorrhage, iron deposits and buried caps **(chapter 2)**. Overexpression of p53 in caps of pre-existing lesions showed a similar transition, which became even more apparent after stimulation with a vasopressor. P53 caused an increase in apoptosis and a concomitant decrease in proliferation, resulting in an unstable cap, poor in SMCs and fibrous tissue[11]. A postulated mechanism for the characteristics of plaque vulnerability may lie with the increased numbers of apoptotic cells[25-27]. Apoptosis of cap cells means loss of SMCs and collagen production, leading to plaque instability[28]. Furthermore, apoptotic endothelial and smooth muscle cells and bodies expose their membrane phosphatidylserines. SMCs thereby acquire thrombin-generating capacity. Tissue factor is increased on the surface of apoptotic cells. A concomitant loss of anti-coagulant membrane components may promote a pro-coagulant environment[29]. If not promptly removed, apoptotic cells may also become pro-inflammatory[3;23;24;29]. Thus, apoptotic cell death of ECs and SMCs is detrimental for the stabilizing function of the cap and can increase plaque vulnerability.

Another way of cell death, *i.e.* necrosis, is pro-inflammatory by definition. Apoptosis, necrosis and secondary necrosis can contribute to the formation of the highly inflammatory and pro-coagulant necrotic core. Composition and size of the necrotic core have been linked to plaque vulnerability[30]. The necrotic lipid core consists of insudated lipid material and cells that fail to clear this debris[30;31]. As the core increases in size also

stress on the cap and matrix increases[32]. There is a higher risk of rupture with cores greater than 30-40% of the plaque volume[33;34]. The inverse relationship of the lipid core and cap thickness[35] suggests that there may be a point where core size supersedes cap strength. TNFα and macrophage specific p53 deficiency can induce a shift from apoptosis to necrosis and stimulate the formation of more advanced lesions, which can be more prone to rupture **(chapter 2)**. Together, apoptosis of macrophages would be preferred to necrosis. Next to the necrotic core, also infiltration of inflammatory cells in the fibrous cap can destabilize atherosclerotic lesions[7]. Macrophages and T-cells infiltrate at the shoulder of the lesion, releasing their pro-inflammatory cytokines and secreting matrix degrading enzymes, thereby weakening the cap in multiple ways[7;8;36]. Ultimately this can result in rupture or related events (**chapter 3**). In conclusion, macrophages contribute to increased plaque vulnerability affecting both the cap and the core of the plaque.

Together, the data from this thesis support that apoptotic and necrotic cell death and inflammation are important processes in atherosclerosis. Both cell death and inflammation may initiate atherosclerosis and can cause progression of the atherosclerotic plaque, ultimately leading to rupture. These processes go hand in hand (Figure 1). Inflammation can induce apoptosis and apoptosis can induce inflammation. The question is: What happens in the atherosclerotic plaque with inflammation without apoptosis and vice versa? A clarification of the molecular mechanisms responsible for vascular inflammation and cell death may aid in the development of novel therapeutic strategies to treat atherosclerosis and its complications, including the acute coronary syndromes.

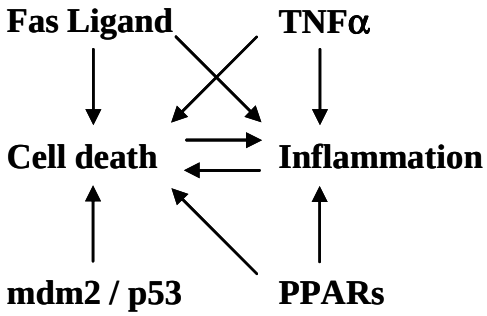


**Figure 1.** Schematic representation of genes described in this thesis and their relation to cell death and inflammation.

***Murine Plaque Rupture Models***

Atherosclerotic complications still account for the major death percentage in the westernized world. In the atherosclerosis research field we made a major step forward with the development of genetically engineered atherosclerotic mouse models, such as mice lacking apolipoprotein E and the LDL receptor, and the ApoE*3-Leiden mouse.

These mice develop reproducible, widespread atherosclerosis and lesion progression that resembles human atherogenesis. However, one of the major drawbacks of these animal models is the lack of end-stage atherosclerosis with spontaneous plaque rupture. Here, plaque rupture is defined as an area of fibrous cap disruption, whereby the overlying thrombus is in continuity with the lipid core[37]. However, present existing models of spontaneous plaque rupture use a broader definition. Several groups have reported a kind of spontaneous plaque rupture in the brachiocephalic artery of apoE$^{-/-}$ mice[38-40]. Observations thus far merely show intra-plaque hemorrhage without thrombosis and not true plaque rupture. Strikingly, apoE$^{-/-}$ mice deficient in scavenger receptor B1 develop severe occlusive coronary lesions resulting in myocardial infarctions at early age[41]. Whether this is the result of true plaque rupture or is caused by excess lipid accumulation or vasoconstriction remains to be determined. Critics argue that mice and humans differ in cholesterol metabolism and lipid profile, cardiovascular physiology, plaque pathology and the plaque progression leading to thrombotic occlusion and clinical events[42-44]. Therefore, although the importance and relevance of an animal model for plaque rupture is undeniable, care needs to be taken in extrapolation of murine to human studies.

In addition to the genetically engineered mouse models of spontaneous plaque rupture, models in which acute plaque rupture is induced mechanically[45-48] or pharmacologically[11;49;50] have been developed. Such models bear little resemblance to human plaque rupture or thrombosis. Other models that have reported thrombosis or myocardial infarction could not prove association or correlation with plaque rupture[51]. In the preceding research to the formation of this thesis, we tried to achieve the development of a plaque rupture model by affecting plaque composition. Similar to the study by von der Thusen *et al.*[11], we overexpressed FasL in the SMC-rich caps of pre-existing plaques. Even in absence of a vasopressor, FasL-induced apoptosis resulted in alteration of the plaques to a more vulnerable phenotype, but without actual thrombotic rupture. Although in this technique the applied incubation pressure is arbitrary, **chapter 3** and the study by von der Thüsen *et al.* could be seen as a first step towards the development of plaque rupture models, in which via the affection of plaque composition rupture-like events occur more frequently and in a controlled fashion. They may help to delineate the molecular pathways involved in plaque (de)stabilization and to evaluate (anti-inflammatory or anti-apoptotic) therapies aimed at plaque stabilization and prevention.

Furthermore, in addition to adenoviral overexpression of cell death inducing ligands we used the site specific recombinase technique[52] to generate several macrophage or SMC-specific conditional mouse models for the deletion of genes involved in cell turnover and inflammation. Studies on the macrophage specific deletion of p53 (Boesten and Zadelaar *et al.*, submitted) and retinoblastoma (Rb) (Boesten and Zadelaar *et al.*, submitted)

showed that p53 deficiency and Rb presence could cause a change in plaque composition possibly leading to a more vulnerable plaque phenotype. Furthermore, the study of **chapter 4**, in which SMC specific deletion of mdm2 was achieved in a similar conditional model, showed dramatic impact on SMCs of the stomach and intestines. Unfortunately, the lethal phenotype of this mouse model upon whole body SMC-specific deletion of mdm2 hampered execution of atherosclerosis studies. Nevertheless, the severity of the phenotype might predict what to expect from mdm2 deletion in the SMCs of the atherosclerotic cap. In conclusion, models that manipulate plaque composition rather than mechanical and pharmacological rupture models resemble human plaque rupture in a better way.

The study in **chapter 3** and the study by von der Thüsen *et al.* that overexpressed FasL and p53 in the SMC-rich cap showed dramatic effects on plaque phenotype, including rupture-like events. On the other hand, the studies on the effects of TNFα **(chapter 2)**, macrophage p53, and Rb deficiency (Boesten and Zadelaar *et al.*, submitted) showed less dramatic effects. This suggests that the targeted location (cap versus core) or cell type (SMC versus macrophage) can determine the effect of a gene in plaque composition and thereby the severity of the phenotype. From the above mentioned studies it is clear that, when targeted in SMCs opposed to macrophages, genes involved in cell turnover have more severe effects on phenotypic alteration. Overall, with this set of genes and these techniques we were only able to induce more or less severe phenotypic changes, but not actual plaque rupture with a clinical end stage.

From large scale gene expression studies performed on whole mount vascular tissue[53-61] it was derived that regulated genes belonged to 3 groups that were already linked with mechanisms in plaque rupture. The mechanisms are 1. a disturbed balance in extracellular matrix turnover, 2. disturbed regulation of cell turnover and 3. processes involved in lipid metabolism[55;62]. The choice of our genes focused on the second major pathway. Our studies, in which we see only more or less severe phenotypic changes by targeting one pathway, support the theory of Faber *et al.* that intervention in one pathway results in less dramatic phenotypes as when at least 2 of these pathways would have been targeted[62]. The multifactorial character of plaque rupture suggests that interventions may be most effective when more than one mechanism at a time is influenced. Our studies have helped us one step further to elucidate the mechanism of affection of plaque composition and thereby plaque vulnerability. Accumulation of knowledge may bring us closer to the development of an actual plaque rupture model, as to date still no good model for plaque rupture exists[42;51;63;64].

***Local Vascular Gene Targeting***

In conventional gene targeting, early embryonic lethality or complex phenotypes often obscure the roles of subject genes at later stages of development or in specific tissues. Therefore, in **chapter 2** we thoroughly checked for any abnormalities as a result of whole body TNFα deletion related with atherosclerosis that could obscure the results from that study. We were not able to find any. In **chapter 3** instead of tail vein injection we chose for local incubation with a FasL expressing adenovirus, avoiding the detrimental death effect that FasL can have on the liver, as a result of the first pass effect. Knocking out Mdm2 and Rb genes in a whole body fashion is lethal and in the case of knocking out p53 tumorigenesis is induced. Conditional gene targeting provides a means to circumvent certain limitations of conventional gene targeting[52]. The development of conditional models was a major step forward in studying cell-type specific effects of a gene and thereby unravelling the underlying mechanisms of disease development. However, there are still several drawbacks. In our case, conditional models drive the costs high, due to high labour-intensity (crossbreeding 3 mouse strains and genotyping) at the expense of a lot of "useless" mice, before getting the right genotype on an atherosclerosis-susceptible background. Eventually, we achieved non-inducible macrophage and inducible SMC-specific mouse models. With the macrophage specific mouse models that were not inducible, and therefore had the macrophage specific deletion from birth on, no abnormalities were observed as a result of absence of the Mdm2, p53 or Rb genes. However, in **chapter 4** concerning the inducible SMC-specific mouse model, we experienced that, although cell type specificity was achieved, lethality was still induced by deletion of Mdm2, hampering studies initially planned on the vasculature. To solve this problem one should apply a cell and tissue-specific promoter that does display a sufficiently narrow expression pattern. Secondly, one could design local application systems to restrict recombination and thereby deletion of a gene to the site of TMX delivery. **Chapter 5** showed that with the unique perivascular delivery device we achieved restriction of recombination of the treated area.

Although the technique used in **chapter 5** allowed studies on the vasculature, recombination levels were not sufficiently high. The low recombination levels are in contrast with initial observations by Kuhbandner *et al.* with the SM22-CreER$^{T2}$(ki) mouse model[65]. By local as well as systemical TMX treatment we achieved maximal recombination levels of 8% in vascular SMCs versus 29% by Kuhbandner *et al.*. Although non-vascular SMC recombination levels were similar, having tried everything to optimise and equalize the protocols, we could not increase the recombination levels of vascular SMCs. One explanation for this phenomenon may be that the activity of the SM22 promoter fragment used in the SM-CreER$^{T2}$(ki) construct is decreased in vascular SMCs

versus gastrointestinal SMCs. However, indirect analysis of SM22 promoter activity by measuring Cre mRNA levels using quantitative real-time PCR did reveal relatively high expression levels in both vascular and gastrointestinal SMCs in our mice. Alternatively, the difference in recombination efficiency between vascular and gastrointestinal SMCs could be caused by differences in accessibility of the loxP sites for the Cre enzyme[66]. Consequently, the limited recombination levels hampered studies with the set of genes of our choice on atherosclerosis. This was supported by the fact that heterozygous deletion of macrophage specific Mdm2 is able to account for complete p53 clearance (Boesten and Zadelaar *et al.*, unpublished data). The only way such low recombination levels might be relevant in loss-of-function or gain-of-function experiments is when specific secretory proteins (cytokines, chemokines, enzymes) are targeted. Recent data on the secretory tissue inhibitor of metalloproteinase-3 (TIMP-3) showed that a 8-10% adenoviral transduction efficiency resulted in potent effects on gelatinolytic activity, apoptosis and vascularization of melanomas[67]. In conclusion, although we had several mouse lines (Mdm2, p53, Rb) ready for research, the low recombination levels with the SM22CreER$^{T2}$ mouse hampered studies with the set of genes of our choice on atherosclerosis. In future experiments at least a different SSR mouse model than that of Kuhbandner *et al.* should be used and if possible a promoter with a sufficiently narrow expression pattern.

## Future Perspectives

Up till now, there is no cure for atherosclerosis or its sequelae. Looking at the bigger picture, atherosclerosis as the main cause of CVD is also the major burden of the metabolic syndrome. The metabolic syndrome (or Syndrome X) is a multi-factorial disease with a cluster of risk factors. The prevalence of the metabolic syndrome is about 25% in the USA (68 million people). Worldwide this is estimated to 115 million individuals. The major characteristics of metabolic syndrome include insulin resistance, abdominal obesity, elevated blood pressure, and lipid abnormalities (*i.e.*, elevated levels of triglycerides and low levels of high-density lipoprotein (HDL) cholesterol). Frequently coexisting conditions put the patient at high risk of developing diabetes, chronic kidney disorders and cardiovascular disease. The cardiovascular burden has indeed been recognised as the tip of an iceberg, in which the immersed part is the preceding clustering of metabolic abnormalities (Figure 2)[68]. In this light, the treatment of atherosclerosis is not a simple task, rather risk management of the metabolic abnormalities to avoid exacerbating the syndrome's features. Although in this thesis only atherosclerosis is studied, it is of key importance to appreciate that the systemic disease, and not only the focal manifestation of atherosclerosis, must be addressed for long-term improvement in outcome of patients with this disease.

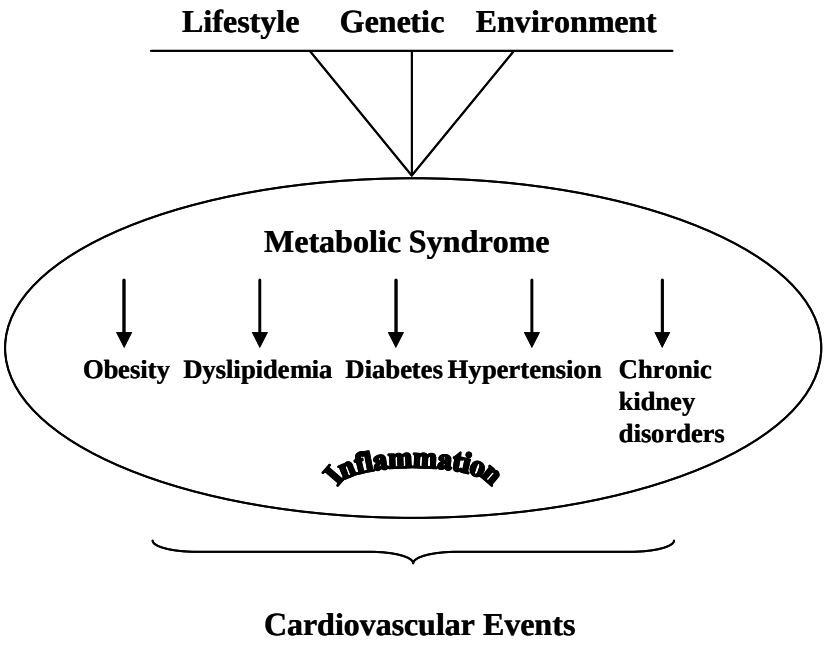


**Figure 2.** Schematic overview of the possible heterogenic and multifactorial origin of cardiovascular events.

Abundant data link cell death and inflammation to atherogenesis. Both processes may be involved in initiation, progression and clinical consequences of atherosclerosis. There is no telling which one is more important, for they go hand in hand. Furthermore, atherosclerosis may result from different origins, including cell death and/or inflammation. Future research could address the question what would happen in the atherosclerotic plaque with inflammation without apoptosis and vice versa? NF-κB is a target that can affect both apoptosis and inflammation[69]. Pharmacological agents such as dexamethasone and tetrapentylammonium (TPA or z-VAD-fmk) can block either inflammation or apoptosis, respectively. Chemically blocking one pathway will reveal effects from the other pathway and vice versa. Identifying the triggers for cell death and inflammation and identifying the details for their pathways may eventually furnish new research and therapeutic areas.

The research of Faber *et al.* supports that with inflammatory and apoptosis genes we are targeting the right set of genes, only targeting one gene at the time will not have major influence[62]. The fact that the atherosclerotic plaque is a multifactorial, highly complicated micro-environment, suggests there is logic in targeting multiple pathways. Future treatment will therefore comprise next to monotherapy, also dual therapy and combination therapy. By example, currently already the cholesterol-uptake blocker ezetimibe is combined with statin treatment, and the insulin-sensitizer metformin is prescribed in combination with rosiglitazone. Therapies aimed at combinations of reducing oxidation and cholesterol accumulation might also exert beneficial effects to reduce initiation,

progression and clinical consequences of atherosclerosis via an actual reduction in inflammation and cell death[28]. Current developmental compounds are targeting dual insulin and lipid regulators, PPAR-pan agonists, and dehydroepiandrosterone (DHEA) analogues. From **chapter 6** we already know that the dual PPARα/γ agonists can act on lipid and glucose metabolism and exert anti-inflammatory and anti-apoptotic actions to our systemical and local benefit on atherosclerosis, which should stimulate good faith in future therapy.

However, treatment in the area of cell death and inflammation can also be complicated. While some components need to be systemically targeted, others would have detrimental effects. NF-κB for instance plays such a central role in the innate and adaptive immunity, and cell death inducing machinery is so conserved in all cells of the body, that whole body targeting could have detrimental effects. **Chapter 4** shows how SMC-specific whole body Mdm2 deletion can have detrimental effects on the intestines and can cause lethality. The data showed that Mdm2 protects intestinal SMCs from p53-mediated cell death with a necrotic morphotype. This study opens new perspectives in a different research field such as cancer and related therapies. The ability of p53 to induce a caspase-3-independent cell death pathway different from apoptosis may be the basis for new therapies to kill cells, in which p53 is wild type, but have acquired defects in the signalling pathways that are downstream p53. In conclusion, from the abovementioned examples it is clear that per targeted gene it should be carefully considered whether local targeting is imperative.

Genes involved in cell death and inflammation need to be targeted at the right place, at the right time, and in the right combination. Conditional models, when optimized, make an excellent tool in this research. A clarification of the molecular mechanisms, responsible for inflammation and cell death in atherogenesis eventually leading to acute clinical events, may aid in the development of novel therapeutic strategies to treat atherosclerosis and its complications, including the acute coronary syndromes. Overall, future research should still be aimed at the design of a physiological plaque rupture model with a clinical end-stage. Considering the multi-factorial and complex situation of plaque rupture, research will have to go a long way to develop a good plaque rupture model. At present, intervention studies will have to investigate one aspect at a time and increase our knowledge until the final breakthrough.

While the specific research on individual processes in the atherosclerotic plaque may favor the accumulation of knowledge, one could question the therapeutic applicability. With the knowledge that we currently possess, a prevention rather than cure approach would be the preferable strategy for the next generation people. At the same time, the effect of elimination of proximal triggers to the benefit of treatment and thereby regression of pre-existing atherosclerosis should be investigated.

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