Optimization of PCR method for analysis of a VNTR in IL-1RN gene for further studies associated with Myocardial Infarction

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Abstract

Atherosclerosis is the major cause of myocardial infarction and is initiated by LDL penetrating the intima of big and middle-sized vessels and converting to oxLDL, followed by migration of T-cells and monocytes, smooth muscle cells, platelets and several immune cells, and the release of pro-inflammatory cytokines, one of which is IL-1β. Interleukin-1 receptor antagonist competitive inhibits the activity of IL-1β. There exists a repeat region polymorphism of an 86 bp segment in intron 2 of the IL-1RN gene, which encodes this anti-inflammatory cytokine, and the allele with 2 copies has been suggested to be associated with atherosclerosis and myocardial infarction.

In this study, an optimization of a PCR-based method for analysis of this polymorphism has been performed, for future analysis of the polymorphism as a risk factor for development of myocardial infarction. Anonymized DNA and DNA from the FIA3 cohort originating from northern Sweden was used in the present study. Samples were amplified with PCR, separated by agarose gel electrophoresis and detected with ethidium bromide/UV-light.

Optimization of agarose concentration, ethidium bromide post-electrophoresis staining, Mg²⁺-concentration and DNA-concentration was performed. The results showed that 2 % agarose gel, 3,0 mM of Mg²⁺ and post-electrophoresis staining were the optimal conditions for detection of 2 and 4 repeats simultaneously when using the Dream Taq DNA polymerase, but no conclusion could be made for the DNA concentration optimization.

Further studies of this polymorphism in the IL-1RN gene are needed to evaluate if it is a risk factor to myocardial infarction.

Key words: Atherosclerosis, inflammation, IL-1RA, tandem repeat region polymorphism, PCR, agarose gel electrophoresis
Abbreviations

CAD .................. Coronary Artery Disease

MI ..................... Myocardial Infarction

LDL.................... Low density lipoproteins

MMP.................... Matrix metalloproteinase

HDL..................... High density lipoprotein

IL-1β.................. Interleukin 1 beta

IL-1RA................ Interleukin 1 receptor antagonist

TNF-α.................. Tumor necrosis factor alfa

SMC.................... Smooth muscle cells

IFN-γ.................. Interferon gamma

TGF-β............... Transforming growth factor beta

PCR.................... Polymerase Chain Reaction
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**Introduction**

**Atherosclerosis**

Coronary artery disease (CAD), including Myocardial Infarction (MI) and heart failure, is the most common cause of death for both men and women [1,2]. In the western world, morbidity by CAD is increasing, mostly because of their lifestyle [3]. The prevalence is significant higher among men. Risk factors for CAD are high age, diabetes, hypertension, obesity, smoking, physical inactivity and some genetic factors. The most common cause of CAD is atherosclerosis, which can start develop even at a young age [1]. It is well known that the build-up of cholesterols and thus, the inflammation process that follows has become a key role in the development of atherosclerosis [4].

Atherogenesis is the development of atherosclerotic plaque, which occurs commonly in the intima of middle-sized and large blood vessels, mostly were they divide due to the disturbed blood flow [1]. *Low density lipoproteins* (LDL) which penetrate into the intima and accumulate (*cholesteryl esters*) are the major cause of atherogenesis [5]. After binding to the proteoglycan matrix, an oxidation/modulation of LDL, possibly the enzyme *lipoprotein-associated phospholipase A2* (*LpPL A2*), occurs and it becomes *oxLDL* which is immunogenic [1,3,6]. Consequently, this leads to activation of the endothelium with expression of adhesion molecules, allowing leukocytes, mostly monocytes and t-cells to bind to and penetrate the endothelium and migrate into the intima [1]. Also, the chemokine *monocyte chemoattractant protein 1* is present in lesions and may guide the migrated leukocytes into the intima [3]. Some dendritic cells, mast cells, neutrophils and B-cells are present in the lesions, however only in a small amount [1]. Platelets migrate to the atherosclerotic plaque, in an attempt to cover the site of damage [6].

The monocytes producing *matrix metalloproteinase 9* (MMP-9) can damage the *extracellular matrix* (ECM), which permits further migration of T-cells into the intima, followed by maturation of monocytes into macrophages and oxLDL binding to toll-like receptors and scavenger receptors, so the macrophages can enclose the oxLDL which eventually turns them into so called *foam cells* [2,3,5]. These cholesterol-contained cells are one of the major causes of the narrowing of the blood vessels which occurs in atherosclerosis. However, some of the foam cells can leave the intima and, by that, export cholesterol from the lesion [3].
addition, there is another known way of which LDL can be removed from the plaque, by the so-called reverse cholesterol transport. The specialized cell-surface molecules **ATP binding cassette (ABC) transporters** mediate a transfer of cholesterol from the foam cells to **high-density lipoproteins (HDL)**, which can transport the cholesterol to **hepatocytes**, where it metabolizes to bile acid and possibly be excreted [3,7,8]. Furthermore, it has been shown that HDL provides with both anti-inflammatory and anti-oxidant properties which may be atheroprotective and be mediated by membrane-bound proteins on macrophages, like paraoxonase [8,9]. For these reasons, HDL is commonly referred to as “good” cholesterol [7]. When the total amount of LDL in the lesion transcends the exported LDL, atherosclerotic plaque will be formed [3].

Macrophages release a number of pro-inflammatory cytokines like **interleukin-1β (IL-1β)** and growth factors like **tumor necrosis factor α (TNF-α)**, both of which stimulate increased expression of leukocyte adhesion molecules on the endothelium such as VCAM-1 and E-selectin, and stimulate production of **fibroblast growth factors, platelet-derived growth factor (PDGF)** and other types of growth factors [3, 10]. Proliferation of macrophages in the lesion may be induced by macrophage colony-stimulating factor, which are largely present in the atherosclerotic plaque [5]. As the time goes by, the accumulation of cholesteryl esters, foam cells and t-cells form a fatty plaque, which narrows the blood vessel and symptoms may be exhibit as **stable angina pectoris or intermittent claudication**. Also, foam cells may undergo **apoptosis** and this leads to a center rich of lipids, called **necrotic core** [3].

Smooth muscle cells (SMC) migrates into the lesion from the media, after changing phenotype into synthetic SMC [1]. On site, SMC are producing extracellular matrix proteins, such as interstitial collagen and proteoglycan. T-cells release pro-inflammatory cytokines, e.g. **Interferon (IFN)-γ**, that can restrict the production of the collagen produced by SMC and stimulate inflammation even further [3,6]. The migration of SMC into the lesion can be induced by PDGF, produced by macrophages, activated platelets and endothelium. Collagen-production by SMC is stimulated by **transforming growth factor β (TGF-β)**, which can be released, by leukocytes, endothelial cells and even from SMC themselves [3]. The production of EMC proteins initiate a conversion of the fatty plaque into a more fibrous one, the so-called **fibrous cap** is formed.
If this fibrous cap ruptures, a thrombosis can occur and may result in a stroke or myocardial infarction [10]. The major cause of plaque rupture is matrix metalloproteases (MMPs), which can degrade the matrix and the cap [11]. Increased expression of these enzymes has been showed to be induced by TGF-β, heparin-binding epidermal growth factor-like factor (HB-EGF) and extracellular matrix metalloproteinase inducer (EMMPRIN) [12]. Monocyte that binds to T-cells can produce and release MMP-9, which is common in atherosclerotic lesions [13]. It is also believed that MMP-8 may have a role in plaque rupture. A way to regulate the production of MMPs is suggested to be a therapeutic way to prevent atherosclerosis [4]. All these mechanisms are shown in figure 1 below, with migrating cells and producing of cytokines, in particular IL-1β, contributes to the unique inflammation which is characteristic for atherosclerosis.

**Figure 1.** The development of atherosclerosis and how it leads to MI or stroke. First, foam cells containing oxLDL accumulate in the tunica intima. With time, smooth muscle cells (SMC) migrate into the lesion and starts to produce ECM proteins like collagen and forming the fibrous cap. Rupture occurs when MMPs are released and this leads to thrombosis and acute conditions like MI or stroke. This picture is originally from [14].
Inflammation

Inflammation is a complex process which is initiated when the body is exposed to adverse or/and foreign agents, damaged cells and sometimes even healthy somatic cells (autoimmunity) [15,16]. Acute inflammation is characterized when pathogens or cells binding to pattern recognition receptors (PRRs), which are activated when bind to Pathogen-associated molecular patterns (PAMPs) or Damage-associated molecular patterns (DAMPs) [3, 17]. When activated, the cells release cytokines and chemokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), which inducing further inflammation and recruitment of more cells. The major pro-inflammatory cytokine IL-1 is produced by monocytes (macrophages), endothelial cells and dendritic cells [10].

IL-1β and IL-1Ra

Interleukin-1 is a pro-inflammatory cytokine that plays a major role in inflammatory diseases, like Rheumatoid Arthritis (RA) and diabetes, and is involved in cell damage and cell proliferation [18]. In addition, IL-1 has been shown to be involved in several mechanisms characterized in Alzheimers disease (AD), for example cholinergic deficit and amyloid deposition [19]. IL-1 binds to two receptors; IL-1R type I and IL-1R type II. Two molecular forms of IL-1 are present in the body: IL-1α and IL-1β, which both are pro-inflammatory but with different functions [18]. In contrast to IL-1α, is basically only an intracellular transcriptional regulator, IL-1β is released by many different cells and is involved in a number of inflammatory mechanisms, including stimulating expression of adhesion molecules on endothelium like VCAM [10,20].

Even though both IL-1 molecular forms are translated as 31kDa molecules without signaling peptide, IL-1α is directly biologically active while the proIL-1β molecule must be cleaved to become activated. This is done by the enzyme caspase 1, also called interleukin converting enzyme (ICE), which is induced by the so called inflammasome, a complex of cytosolic proteins located in a number of immune cells [10, 21]. Different inflammasomes have been found and the most studied is the NLRP3 inflammasome [21]. This can be activated by cholesterol crystals, and therefore could be involved in atherogenesis [22].
Furthermore, there is one anti-inflammatory molecule that competes with IL-1 for binding to IL-1RI; *interleukin-1 Receptor Antagonist* (IL-1Ra), and thus regulates IL-1 signaling [23, 24]. It has been shown in many studies that there is an association between inflammatory diseases, like atherosclerosis, and serum level of both IL-1β and IL-1Ra [25]. The balance of these two has been of high interest among medicine in recent years, e.g. in atherogenesis [26]. If the ratio of IL-1β: IL-1Ra serum level is high, or vice versa, then the patient will exhibit a more inflammatory condition [23,26]. During an acute inflammation, the level of IL-1Ra is normally increased to prevent development of a chronic inflammation [23]. All of the three molecules mentioned above are encoded within a gene cluster located in chromosome 2q14 [27]. Recent studies on polymorphisms located in this gene cluster have been performed to discover any association to diseases, especially on polymorphisms in IL-1β gene, but recently the gene for IL-1Ra (IL-1RN) has been of highly interest [23].

A tandem repeat region polymorphism is located in intron 2 of IL-1RN, which consists of 86 base pair segments and affects the expression of IL-1Ra and the activity of IL-1β [26,28]. Five different alleles have been discovered, with 2-6 copies of the repeat segment [27]. Most common allele is the one with 4 repeats (≈ 74 %), and the second most common is the one with 2 repeats (≈ 21 %) [26,27,28]. The other 3 alleles, with 3, 5 and 6 repeats, have a total prevalence of < 5 % [27]. Because this repeated segment contains three potential protein binding sites, which are an α-interferon silencer A, a β-interferon silencer B and an acute phase response element, may be the number of repeats influences the function of this gene/cytokine significantly [28].

Recent studies have shown that the 2 repeat-allele is more common among patients with several different inflammatory diseases, including *systemic lupus erythematosus* (SLE), *ulcerative colitis* (UC), *Rheumatoid Arthritis* (RA) and *diabetes nephropathy*. Up regulation of IL1RN was found in atherosclerotic plaque and the 4-repeat allele was associated with larger lesion size compared to carriers of the IL-1RN 2-allele [26]. Also, several studies concluded that the 2 repeat-allele was associated with higher risk for gastric cancer development [29, 30]. Furthermore, an association between this allele and *Coronary artery disease* (CAD) has been shown [31], and it has been suggested that the 2-repeat allele is connected to
development of atherosclerosis and thereby myocardial infarction. However, the role of IL-1RN in myocardial infarction is not clear.

**Aim:**

In the present study we aim to optimize a PCR-based method for the analysis an 86 base pair long tandem repeat polymorphism in intron 2 of IL-1RN gene, and to further analyze if this polymorphism is a risk factor for the development of MI.
Methods and Materials

Ethics

The study has been ethically approved by the local research ethics committee of Umeå University.

Materials and methods

For the optimization procedure, anonymized DNA samples were used. DNA from the FIA3 cohort was collected from Northern Sweden. The samples were stored as 10 ng of dried DNA/well. The samples were dissolved in 20 µl dH₂O, making the final DNA concentration 0,5 ng/µl. From this 0,5, 2,5 and/or 5,0 ng was obtained and used during the optimization.

PCR

For the optimization, 0,5-5 ng of FIA3 DNA or 1-10 µl of anonymized DNA was amplified in a 20 µl reaction containing 1x Dream Taq DNA polymerase buffer (Thermo Scientific, Waltham, Massachusetts, USA), 1 µM of each primer (IL-1RA F Tarlow: 5’-CTCAGCAACACTCCTAT-3’, IL-1RA R: 5’-TCCTGGTCTGCAGGTAA-3’; Invitrogen, Carlsbad, California, USA), 200 µM of each dNTP (Invitrogen) and 0,5 U DreamTaq DNA polymerase (Thermo Scientific). In the optimization procedure, 2,0 mM, 2,5 mM, 3,0 mM or 4,0 mM of Mg²⁺ were used. For the PCR (Whatman Biometra, Tgradient, Göttingen, Germany), a profile with this setup was used; hot start at 94 °C during 2,5 min, after which 36 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 2 minutes were performed with a final extension at 72°C during 7 min, before the samples were stored at 18°C until gel electrophoresis analysis.

Agarose gel electrophoresis

For the gel electrophoresis, agarose (Agaros Standard, Saveen Werner AB) gels with a concentration of 1,5 % or 2,0 % diluted in 1xTBE were used. The gels underwent electrophoresis (BioRad, Power Pac 300, Hercules, California, USA) at 120 V during approximately 30-45 min followed by a visual analysis under UV-lamp (Techtum Lab AB, Umeå Sweden) with a camera (CCD, Video Camera Module, Japan) and printing (Mitsubishi P91, Tokyo, Japan) of pictures.
Results

Optimization of agarose gel concentration and Ethidium Bromide post-analysis staining

Firstly, a comparison between samples transferred and visualized in two gels with different agarose concentrations (1.5 % vs 2 %) was performed. As figure 2 and figure 3 below shows, there was in general a slight difference for the separation of the samples between respective agarose concentrations, with 2 % agarose gel exhibiting the best separated bands with a well-separated ladder. For continued optimization was the 2 % agarose gel used.

We also investigated if the samples needed extra ethidium bromide staining post-agarose gel electrophoresis. In figure 3A and 3B, the gel was stained for 20 min in 1xTBE buffer with ethidium bromide. It’s harder to distinguish the 2-repeat allele in figure 2A and 2B (samples 6, 8, 12 and 14) without post-electrophoresis staining, while a homozygote for this allele is easy seen in figure 3 B (sample 51) and the 2-repeat alleles from the three heterozygote samples in figure 3 A (samples 38, 40 and 41) is relatively easy to distinguish when using post-electrophoresis staining. Therefore, it was decided that post-electrophoresis Ethidium Bromide staining was better for further optimization.

**Figure 2 A (left) and 2 B (right).** PCR products separated on a 1.5% agarose gel. Anonymous DNA samples were amplified and loaded onto a 1.5 % agarose gel with EtBr but without post-electrophoresis staining. The bands with 4 repeats are 410 bp and the ones with 2 repeats are 240 bp. The 2-repeat bands were evident in sample 6, 8, 12 and 14 (arrows). However, this was easier seen in the original printed pictures.
Optimization of Mg$_2^+$ concentrations

In addition, four different concentrations of MgCl$_2$ in the master mix were compared; 2.0 mM (standard concentration), 2.5 mM, 3.0 mM and 4.0 mM, for three different genotypes: 4-repeat allele homozygous (sample 44), 2-repeat allele homozygous (sample 40) and heterozygous for both alleles (sample 24). These were decided to be used and as representative for these genotypes.

A pronounced difference was seen between the standard (lowest) concentration of Mg$_2^+$ compared with all the other concentrations. For each increase of the Mg$_2^+$-concentration, the bands got slightly brighter, except for the highest concentration (4.0 mM) where, evidently, non-specific bands appeared. As seen in figure 4 below, these bands makes it seem like sample 44 and 24 are heterozygous when using 4.0 mM of Mg$_2^+$, rather than 4-repeat homozygous and 2-repeat homozygous, respectively. Also, a possible non-specific band is exhibited below the 4-repeat allele for the heterozygous sample 40 when using 4.0 mM MgCl$_2$. In general it seems like the second highest concentration (3.0mM) exhibited the most distinct and clear bands for all the three genotypes, in particular for the 2-repeat homozygote and the 2-repeat allele for the heterozygotes, which were barely visible when using 2.0 or 2.5 mM Mg$_2^+$ as seen in figure 4. Considering this, it was decided that 3.0 mM MgCl$_2$ should be used for further optimization.

*Figure 3 A (left) and 3 B (right). J. PCR products separated on a 2 % agarose gel. Anonymous DNA samples were amplified and loaded onto a 2 % agarose gel with EtBr. Post-analysis staining has been used. The bands that are 410 bp long have 4 repeats while the bands that are 240 bp long have 2 repeats, which the black arrows indicate. White arrows indicating where the allele with 2 repeats (sample 38, 40 and 41 as heterozygotes, and sample 52 as homozygous for the 2-repeat allele) are located.*
Optimization of DNA concentration

Several experiments with FIA3 samples and previous analyzed anonymized DNA as positive controls were performed with the amplification of 0.5, 2.5 or 5 ng DNA which came from the FIA3 cohort were utilized.

It was easier to see the bands for the samples with 2.5 ng DNA compared to those with 0.5 ng DNA, which is shown below in figure 5A and B. However, the negative control was contaminated in the samples with 0.5 ng (figure 5A). However, no conclusion should be drown of which DNA concentration is better due to the contamination, even if the bands were brighter and stronger with 2.5 ng of DNA.
Figure 5 A (left) and 5 B (right). Five FIA3 samples (A1-A5), three anonymized controls (46, 51, 52) and negative controls (NC) are shown in these two pictures. Figure A represents 0.5 ng of DNA and in figure B 2.5 ng of DNA have been amplified. The black arrows indicating the respective exhibited alleles. The negative control (NC) in figure A is contaminated (arrow).
Discussion

During this study, an optimization of classical PCR combined with gel electrophoresis for a tandem repeat region polymorphism in the IL-1RN gene was performed. Optimization of the annealing temperature had already been performed and it was concluded that 59°C was the best choice. This temperature was used as annealing temperature during this study. The annealing temperature is specific for each different oligodeoxynucleotide primer used and different polymorphisms have their own optimal annealing temperature. Annealing temperature is usually somewhere within 55-65°C [32].

The first step was to compare samples loaded onto two gels with the agarose concentrations of 1.5% and 2%. The best separated samples were seen with 2% of agarose, with an easier readable ladder. It is highly important that the samples have separated enough to be able to distinguish between the two alleles. Although this study showed that 2% agarose is the optimal concentration for this polymorphism, this is not that surprising because small DNA molecules like this (240 or 410 bp) usually separate fastest when using high concentration gels, while bigger DNA samples (>1kb) separate better in gels with low concentrations [32]. Previous studies have used both 1.5% [26] and 2.0% [28,31] agarose gel for analyzing this polymorphism, but according to this study, 2% of agarose is optimal.

The results showed that the samples in both gels which exhibit the 4 repeat-allele were considerably easier to see, but the gel which underwent post-electrophoresis EtBr staining (figure 3) may had slightly stronger bands for this allele. However, the bands representing the 2 repeat-allele in the gel with no post-electrophoresis staining were barely visible to the naked eye, whereas they could be distinguished relatively easy in the gel with post-electrophoresis staining. It tended to be considerably more difficult to see the bands for the 2-repeat allele than the bands for the 4-repeat allele, in particular for heterozygous samples. Why this occurred is likely because the 4-repeat is considerably longer and thereby it has more spots where the ethidium bromide can bind to, which lead to more fluorescence and brighter bands for the 4-repeat allele compared to the 2-repeat allele. Thus, the strength of the 2-repeat allele for heterozygotes would be the major crucial factor when determining if the method is optimal or not. Although EtBr is usually an effective staining agent, it is mutagenic and carcinogenic, and should therefore be used with great caution and the spot
where it has been used should be cleaned properly. Some other stainings for amplified DNA or RNA are available, such as GelRed and SYBR Green, however none of these is decided that these isn´t as effective with regard to all the factors as EtBr, even with the health dangers in consideration [32]. Due to our results, the best choice of option is to perform post-electrophoresis staining.

An optimization of Mg\(^{2+}\)-concentration was made, comparing the standard concentration (2,0 mM) with 2,5 mM, 3,0 mM and 4,0 mM MgCl\(_2\), for three different samples with different genotypes; homozygous of the 4 repeat-allele, homozygous of the 2 repeat-allele and for heterozygous of both alleles. These samples were chosen based on the bands they exhibit from a previous amplification in this study. Magnesium ions are stabilizing the enzyme Taq polymerase at high temperatures, making it dependent on Mg\(^{2+}\) to be able to work properly [32]. The results showed stronger bands with increasing Mg\(^{2+}\) concentration, except at the highest concentration which exhibited bands at the level representing the 2 repeat-allele in homozygotes of the 4 repeat-allele and bands where exhibited at the level for 4 repeat-allele in homozygotes of the 2 repeat-allele. This is easily explained by the fact that excess of Mg\(^{2+}\) will create non-specific bands [32], which is worth taking in consideration. However, cautious interpretation of our results should be done, since the band exhibited at the level of the 4-repeat allele for sample 24 when using 4,0 mM in figure 5 is certainly non-specific, given that, as mentioned above, the 4-repeat allele should bind to more ethidium bromide and thus be brighter than the 2-repeat allele, even without any addition of Mg\(^{2+}\). The band that appears in the 4,0 mM Mg\(^{2+}\) concentration for sample 24 at approximately 400 bp is therefore most likely unspecific. Furthermore, if sample 44 would be heterozygous, then the second highest Mg\(^{2+}\)-concentration (3,0 mM) should have exhibited at least a diffuse band at the level for the 2-repeat allele, given that this concentration exhibit a band on this level for the heterozygous sample 40. However, to verify our results, sequencing of the controls would be the next step. But different DNA polymerases are more or less sensitive to lower or higher concentrations of Mg\(^{2+}\) [32]. With regard to this, 3,0 mM of MgCl\(_2\) would be the best choice when analyzing this polymorphism with the Dream Taq DNA polymerase.

For the optimization of the amount of FIA3 DNA, DNA concentrations of 0,5, 2,5 and 5,0 ng were analyzed and compared for optimal results. Several amplifications were performed for
the two lower concentrations, but only one with 5,0 ng DNA, due to limited amount of DNA in the FIA cohort. In addition, if the negative control would be contaminated, then valuable DNA samples from the FIA-3 plate would be used in vain. There were stronger and brighter bands when using 2,5 ng DNA than using 0,5 ng DNA. However, a band at the level for the allele with 2 repeats was present in the negative control with the lowest DNA-concentration, which influences the interpretation of the results given that the DNA showed in figure 5 were both from same individuals. Most of the amplifications performed with 0,5 ng DNA exhibited visible bands in the negative control except two, but these exhibited in general barely visible bands for the DNA samples and the samples used in these were not the same as those used with 2,5 ng of DNA. Thus, a comparison between any of those two samples with 0,5 ng DNA and the samples with 2,5 ng would not be reasonable. After several failed attempts to get a negative control without contamination, it was decided that the experiments were terminated due to time limitations.

Initially, the study was supposed to analyze a number of 96 wells FIA3-samples to assess if the 2-repeat allele might be a risk factor for development of atherosclerosis and hence MI. A previous study in our research group showed that patients who carried the 2-repeat allele were associated with a smaller mean plaque area than the 4-repeat homozygous patients from another MI cohort, called the SCARF cohort [26]. In the present study, we therefore intended to increase the amount of samples, why the FIA3 cohort was selected. The optimization procedure with the patient samples however failed, since bands in the negative control appeared and due to time limitations, we could unfortunately not proceed. Therefore, no genotype or allele frequencies for the polymorphism can unfortunately be displayed. To avoid this in future studies, it is highly important to have a clean and sterile working spot and materials being used. Also, the technique when preparing the master mix and adding the DNA samples is something to think about.

The frequency of the different alleles differ between different ethnic groups. Previous studies of this polymorphism in different European cohorts found that the frequency of the 2-repeat allele among healthy controls was the following: in Germany: 27% (n=234), Italy: 23% (n=458), Spain: 30% (n=130), Belgium: 24% (n=401) and Finland: 28% (n=381), while the frequency of the 4-repeat allele in the same studies was the following: in Germany: 69%, Italy: 74%, Spain: 68%, Belgium: 74% and Finland: 71%. [33-37]. In addition, two studies
showed that the frequency of the 2-repeat allele is lower among Black African (12%) and African American (12%) healthy controls compared to the White African (25%) and Caucasian (46%) healthy controls. Also, the frequency of the 4-repeat allele was higher for black African (87%) and African American (87%) than White African (73%) and Caucasian (90%) [38,39]. Furthermore, studies have shown that the frequency of the 2-repeat allele is even lower in east Asian populations, including Thailand (10%), Taiwan (6%) and Korea (3%) [40,41,42]. Although the Swedish population is under genetic drift [43], it is likely that the allele frequency of the 2-repeat allele should be between 25-30% and the 4-repeat allele should be approximately 70% for the normal population of the FIA3 cohort.

Several studies have shown an association between the 2-repeat allele in IL-1RN and inflammatory diseases like diabetes nephropathy, rheumatoid arthritis, SLE, and also coronary artery disease (CAD). One study in particular, concluded that a higher risk for single-vessel coronary artery disease (SVD) was associated with the 2-repeat allele of this, however multi-vessel coronary artery disease did not show any significant difference compared to the control samples [36]. Also, Olofsson, et al. showed that the balance between IL-1Ra and IL-1β was shifted toward a more pro-inflammatory condition for atherosclerotic patients than healthy patients [26]. This cohort consisted of only 387 post-MI samples, why we wanted to verify these data in a larger cohort, such as the FIA3 cohort. Further studies of this polymorphism are needed to evaluate the possibility as a risk factor for developing atherosclerosis and myocardial infarction.

In conclusion, the optimal concentration of agarose in gels for analyzing this 86 bp long tandem repeat region polymorphism in IL-1RN gene is 2%. In addition, a 3mM Mg²⁺-concentration gave the strongest and most reliable results when using the Dream Taq DNA polymerase. Furthermore, post-electrophoresis ethidium bromide staining gave the best results.

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