UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Functional role of microRNAs in acute coronary syndrome patients younger than 45 years old

Papel funcional de los microRNA en pacientes de síndrome coronario agudo menores de 45 años

Ascensión María de los Reyes-García Pastor

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Autora: **Ascensión María de los Reyes-García Pastor**

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Functional role of microRNAs in acute coronary syndrome patients younger than 45 years old

Papel funcional de los microRNA en pacientes de síndrome coronario agudo menores de 45 años

y dirigida por,

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A mi abuelo Antonio,
el más incansable investigador
que he conocido nunca.

A mi madre,
a mi padre,
a mi hermana,
por todo.

RESUMEN

Las enfermedades cardiovasculares son un problema de salud pública en todo el mundo, y las cifras indican que su incidencia no disminuirá a medio plazo. En Europa, por ejemplo, se reportaron aproximadamente 4 millones de muertes en 2021 asociadas a estas patologías. La modalidad más frecuente es el infarto agudo de miocardio, una de las presentaciones clínicas del síndrome coronario agudo (SCA). La edad es un factor de riesgo importante en esta enfermedad, siendo la media del primer evento de entre 65 y 70 años y la causa más frecuente, la rotura de una placa aterosclerótica. Sin embargo, el SCA también puede ocurrir en pacientes jóvenes. Los pacientes menores de 45 años representan alrededor del 6% del total, presentan factores de riesgo y manifestaciones clínicas diferentes, considerándose un grupo diferenciado; un 20% de estos pacientes jóvenes no tiene aterosclerosis. En los últimos años, y en paralelo con el desarrollo tecnológico, se ha puesto de manifiesto que la enfermedad cardiovascular es una entidad en la que interaccionan la inflamación, la inmunidad innata y la coagulación, por lo que el modelo de etiología única está obsoleto. En pacientes más jóvenes no se conoce bien el alcance fisiopatológico de estos procesos, por lo que es especialmente útil analizarlos para identificar nuevos biomarcadores pronósticos y predictivos.

En esta interacción entre inmunidad, inflamación y coagulación tienen un papel fundamental los neutrófilos y las trampas extracelulares de neutrófilos (NET) que liberan. Los NET están formados por redes de ADN cubiertas de histonas y proteínas citosólicas, como mieloperoxidasa o elastasa, y son importantes efectores del proceso de inmunotrombosis. La NETosis es un proceso fisiológico de la inmunidad innata intravascular para proteger al organismo contra los patógenos invasores, que cuando se desequilibra puede provocar trastornos trombóticos. Además de su función bactericida, los NET también provocan una potente respuesta procoagulante, iniciada principalmente con la unión y activación de las plaquetas. Los componentes de los NET representan una nueva clase de patrones moleculares asociados al daño (DAMP) que puede activar la inmunidad innata y, por tanto, podrían resultar útiles como biomarcadores en SCA.

Otras moléculas que cumplen todos los requisitos para ser un biomarcador ideal en esta enfermedad son los microRNA (miRNA). Se trata de moléculas de ARN de aproximadamente 20 nucleótidos, que no codifican proteínas, pero son capaces de regular su expresión. Se encuentran presentes en sangre, orina y otros fluidos, lo que los hace fácilmente accesibles, además son resistentes incluso a condiciones extremas,

por lo que suponen un útil biomarcador no invasivo. La importancia de los miRNA en la aparición de diversas y numerosas patologías ha sido bien establecida, por lo que esta Tesis se centra en el estudio de variantes en genes de miRNA relacionados con los procesos inflamatorios y aterotrombóticos propios del SCA en pacientes menores de 45 años.

Hipótesis: El SCA es una enfermedad compleja, en cuyo desarrollo intervienen factores epidemiológicos y hábitos adquiridos, con una plausible participación de alteraciones genéticas, aunque estas últimas han sido menos estudiadas. Tradicionalmente, el SCA ha sido considerado una enfermedad asociada a la edad, con un desarrollo paralelo al envejecimiento del individuo. Esta circunstancia implica una base fisiopatológica para un proceso inflamatorio sostenido, la aterosclerosis, de crucial importancia en el SCA. Los pacientes más jóvenes, sin embargo, se consideran un subgrupo clínico diferenciado en el que la erosión de la placa, más que la aterosclerosis, puede jugar un papel relevante. Sus características distintivas se pueden asociar a una presentación clínica, pronóstico, evolución y tratamiento diferente. En paralelo al desarrollo tecnológico, en los últimos años se ha identificado la participación de muchos tipos celulares diferentes en las placas ateroscleróticas, incorporándose la noción de que la inmunidad innata también juega un papel en su progreso. En este contexto, la visión más innovadora de la enfermedad cardiovascular contempla la interacción de tres procesos, inflamación, inmunidad innata y hemostasia, definidos en su conjunto como inmunotrombosis. Estos nuevos conceptos se han establecido en varias series de pacientes mayores, pero no se sabe si están igualmente implicados en pacientes más jóvenes con SCA. Nuestra hipótesis es que el SCA en pacientes jóvenes es también una enfermedad con un importante componente tromboinflamatorio. Además, una vez controlados los factores de confusión inherentes al envejecimiento, la implicación de los factores genéticos será más evidente. En la búsqueda de alteraciones genéticas buscamos variantes en los genes de los miRNA que, al ser reguladores de procesos inflamatorios y aterotrombóticos, pueden estar asociados a la aparición, gravedad y evolución del SCA en pacientes más jóvenes.

Objetivos: Como objetivo central de esta Tesis Doctoral se planteó estudiar si el SCA en pacientes jóvenes es una enfermedad tromboinflamatoria, e identificar nuevas variantes genéticas en genes de miRNA que puedan ser útiles como biomarcadores de tromboinflamación, desarrollo y/o recurrencia de la enfermedad en estos pacientes.

Objetivo 1: Estudiar la asociación del genotipo rs2431697 de miR-146a con la producción de trampas extracelulares de neutrófilos (NET) en relación con la ocurrencia de un SCA y con el curso clínico en pacientes jóvenes (≤45 años).

Objetivo 2: Investigar el potencial trombogénico de la histona H4 y el ADN (componentes esenciales de los NETs) analizando su efecto en la expresión de factores de la coagulación y determinando si estos cambios de expresión pudieran estar regulados por miRNA.

Objetivo 3: Identificar nuevas variantes en genes de miRNA con un potencial patológico en el SCA en pacientes jóvenes.

Material y métodos: Para alcanzar estos objetivos se dispuso de muestras de plasma de pacientes de ACS menores de 45 años en el momento del primer evento, recogidas de manera retrospectiva, así como de controles sanos. También se aisló ADN total de todos los sujetos incluídos. Los pacientes tuvieron un seguimiento de 2 años tras la recogida de muestra durante el cual se registraron todos los eventos adversos. Se consideraron como *end-point* los siguientes eventos: eventos isquémicos incluyendo infarto de miocardio y trombosis del stent, hospitalización por complicaciones cardiovasculares y cualquier causa de mortalidad. Para el estudio experimental, se realizaron cuantificaciones en el plasma, tanto de ADN libre (cfDNA), mediante ensayos fluorimétricos con Sytox, como de histona H3 citrulinada en colocalización con DNA (citH3-DNA) que representa un marcador específico de NET, mediante realización de ELISA tipo sandwich. Para la realización de experimentos *in vitro* se utilizaron cultivos celulares inmortalizados como HepG2, modelo hepático, THP-1, como modelo de monocitos y EA.hy926, como modelo endotelial y se purificó DNA de neutrófilos humanos mediante gradiente de histopaques. También se llevaron a cabo diversas técnicas de biología molecular como purificaciones de DNA genómico y de RNA. El genotipado para el rs2431697 de miR-146a se llevó a cabo mediante sondas Taqman, el genotipado de las dos variantes descritas en el capítulo 3 se llevó a cabo mediante tecnología KASP y los niveles de expresión de RNA mensajero fueron cuantificados mediante qRT-PCR. También se realizó edición génica mediante CRISPR-Cas9. Las

variantes encontradas mediante NGS fueron filtradas mediante una herramienta creada específicamente para tal fin y se establecieron unos rigurosos criterios de selección. Los datos obtenidos se analizaron empleando en cada caso las pruebas estadísticas más adecuadas, utilizando bien el programa SPSS versión 25.0, o bien, GraphPad Prism 9.

Resultados:

Capítulo 1. Tanto cfDNA como los complejos citH3-DNA se encuentran significativamente aumentados en los pacientes jóvenes con SCA en comparación con los donantes sanos, indicando un aumento de NET en estos pacientes. El cfDNA se encuentra en una proporción similar entre los diferentes grupos de pacientes, mientras que citH3-DNA, aunque se mantiene más alto que en controles sanos, disminuye con el tiempo tras el SCA. Encontramos que el genotipo del rs2431697 de miR-146a no tiene relación con la aparición de la enfermedad en nuestra cohorte, aunque los pacientes portadores del alelo T tienen unos niveles significativamente mayores de cfDNA y de complejos citH3-DNA en comparación con los pacientes con genotipo CC. Se demuestra así, por primera vez, que el genotipo del rs2431697 está involucrado en la generación de NET en plasma de pacientes jóvenes con SCA. Por último, se encontró que aquellos pacientes con los niveles más altos de NET en plasma (por encima de la mediana) y portadores del alelo T de rs2431697, presentaban un mayor riesgo de sufrir eventos isquémicos recurrentes.

Capítulo 2. Exploramos el potencial trombogénico de la histona H4 y del ADN, componentes de los NET. Observamos que la histona H4 y, en menor medida, el ADN provocan un aumento en la expresión de RNAm de *F7, F12* y *SERPINF2* (que codifica la α2-antiplasmina), mientras que los niveles de RNAm de *F5, PROS1* y *SERPINC1* (que codifica antitrombina) sólo aumentan con la activación mediante histona H4. Además, ambos activadores provocan un aumento en la expresión de *HNF4A*, un factor de transcripción hepático común a los factores de la coagulación, indicando su implicación en la regulación de factores de la coagulación mediados por componentes de los NET. Por último, se demostró que la histona H4 puede producir una disminución de la expresión de algunos miRNA del cluster miR-17/92, explicando en parte la regulación de la expresión de factor tisular provocada por la H4, mientras que el ADN actuaría de manera independiente al cluster miR-17/92, ya que no es capaz de modificar su expresión.

Capítulo 3. Se analizaron un total de 1175 variantes localizadas en 365 genes de miRNA, y de entre ellas, se seleccionaron 10 variantes con potencial papel patológico. Estas 10 variantes debían encontrarse en genes de miRNA que tuvieran descrita previamente alguna relación con enfermedad cardiovascular o rutas relacionadas con la función cardiovascular. Se estudió la frecuencia de 9 de estas 10 variantes en una cohorte de 300 pacientes jóvenes con SCA y 300 donantes sanos. Tras el genotipado de estas cohortes, se encontraron dos variantes de un solo nucleótido presentes en una frecuencia significativamente más alta en pacientes con SCA que en controles sanos, demostrando un potencial rol como marcador genético de la enfermedad. Ambas variantes se encuentran localizadas en genes de miRNA que han sido previamente relacionados con enfermedades cardiovasculares.

Conclusiones: Este proyecto aporta información novedosa sobre el papel de los miRNA y sus variantes genéticas en el SCA como biomarcadores de la severidad y desarrollo de la enfermedad, e incluso como efectores funcionales de la misma.

1. Los datos presentados muestran que en pacientes jóvenes, al igual que sucede en los mayores, la tromboinflamación es un estado patológico subyacente al SCA. La medición de los componentes de NETosis en plasma parece ser un marcador útil en estos pacientes. Describimos aquí, por primera vez, que el miR-SNP rs2431697 de miR-146a podría actuar como un factor de riesgo adicional de peor pronóstico en pacientes con ACS menores de 45 años. Se propone que el alelo T de rs2431697 puede establecer un estado proinflamatorio silencioso que, al alcanzar el umbral adecuado, puede predisponer a una mayor producción de NET y, por lo tanto, promover una mayor tasa de eventos cardiovasculares adversos.

2. Los resultados encontrados indicaron que la histona H4 y, en menor medida, el ADN, provocan un aumento en la expresión de varios factores de la coagulación. Esta regulación puede alterar el equilibrio hemostático y explicar el efecto protrombótico de estos componentes de NET. Además, tanto el ADN como la histona H4 desencadenaron un aumento en la expresión del ARNm de *HNF4A*, lo que puede indicar su participación parcial en la regulación de los factores de la coagulación mediados por los componentes de los NET. Además, se determinó que la histona H4 podría inducir una disminución en la expresión de ciertos miRNA del cluster miR-17/92, lo que podría explicar la sobrerregulación de la expresión de factor tisular inducida por H4, mientras que el ADN actuaría independientemente de miR-17/92.

3. En este estudio se analiza por primera vez la presencia de mutaciones en los genes de miRNA relacionados con la aparición de SCA, describiendo dos SNV raros que se expresan con una frecuencia significativamente mayor en la población joven de SCA en comparación con la población sana. El papel potencial de estas dos variantes en genes de miRNA como marcadores genéticos de la enfermedad, su implicación funcional y su utilidad como nuevas dianas terapéuticas en la enfermedad cardiovascular, necesitan de una evaluación más profunda y posterior confirmación.

ABSTRACT

Cardiovascular diseases are the main cause of death in Europe with approximately 4 million deaths in 2021. The most frequent cardiovascular disease is acute myocardial infarction, one of most common clinical presentations of acute coronary syndrome (ACS). Patients under 45 years of age represent around 6% of the total. They present their own risk factors and clinical manifestations, becoming a highly differentiated group of patients. Indeed, about 20% of these young patients do not present atherosclerosis. Prompt and accurate diagnosis is crucial for the clinical management of patients with ACS. With the onset of the disease, various factors are released into the blood that can be used as potential biomarkers. In the case of young patients, it is especially useful to find new prognostic and predictive biomarkers.

General aim: To study whether ACS in young patients is a thromboinflammatory disease and to identify new genetic variants in microRNA genes that may serve as biomarkers of thromboinflammation, disease development, and recurrence in young patients. **Objective 1:** To study the association of the miR-146a rs2431697 genotype with the clinical development of young ACS patients through excessive production of neutrophil extracellular traps (NETs). **Objective 2:** To investigate the impact of histone H4 and DNA in the expression of coagulation factors and determine whether the overexpression of tissue factor in response to NET components, histones and DNA, is regulated by microRNAs. **Objective 3:** To identify new variants in microRNA genes with a potential pathological role in the development of ACS.

Material and methods: To achieve these objectives, we retrospectively collected plasma samples from ACS patients under 45 years of age, as well as from healthy controls. For the experimental study, plasma quantification of cell free DNA (cfDNA) and citrullinated Histone H3 in colocalization with DNA (citH3-DNA) were performed. To carry out *in vitro* experiments, cell cultures of HepG2, THP-1, and EA.hy926 were used. Various molecular biology techniques such as nucleic acid extraction, RT-qPCR, NGS and gene editing by CRISPR-Cas9 were also carried out. The data obtained were analyzed using the most appropriate statistical tests in each case.

Results: 1. NET markers are significantly increased in young ACS patients compared to healthy donors. Patients carrying the T allele of rs2431697 have significantly higher levels of cfDNA and citH3-DNA complexes compared to CC patients. Finally, it was found that the patients in the recurrence group with plasma NET levels above the median and carriers of the T allele, had a higher risk of suffering recurrent ischemic events.

2. Histone H4 and, to a lesser extent, DNA cause an increase in the expression of various coagulation factors. Furthermore, both activators cause an increase in the expression of *HNF4A*. Finally, it was shown that histone H4 can cause a decrease in the expression of some miR-17-92 cluster microRNAs, partly explaining the regulation of tissue factor expression caused by histone H4, whereas DNA would act independently of miR-17-92.

3. Two single nucleotide variants (SNVs) were found to be present at a significantly higher frequency in ACS patients than in healthy controls, they were both located in the area of microRNA genes that have been previously associated with cardiovascular disease, demonstrating their potential role as a genetic prognostic marker of the disease.

Conclusions: This project provides new and valuable information on the role of microRNAs and their genetic variants in ACS as biomarkers of the severity and development of the disease, and even as functional effectors.

1. Plasma NET markers are a useful marker of ACS in young patients. The T allele of rs2431697 may act as an additional risk factor for a worse prognosis in these patients.

2. Histone H4 and DNA can impact the expression of some coagulation factors. Histone H4 also decreases the expression of miR-17-92 cluster, which has been shown to regulate tissue factor. DNA also regulates tissue factor expression, but independently of miR-17-92.

3. We analyzed for the first time the presence of mutations in microRNA genes potentially related to ACS, describing two SNVs present at a significantly higher frequency in young patients than in healthy controls, both located within the gene of a microRNAs described to be involved in cardiovascular diseases.

INDEX

ABBREVIATIONS

ABBREVIATIONS

ACC – American College of Cardiology
ACS – Acute Coronary Syndrome
ACTB – β-Actin
ADP – Adenosine Diphosphate
AGO – Family of Proteins Argonaute
AHA – American Heart Association
AMI – Acute Myocardial Infarction
ASA – Acetylsalicylic Acid
ATCC – American Type Culture Collection
BNP – B-type Natriuretic Peptide
bp – Base-pair
Cas – CRISPR-Associated Endonucleases
C1INH – C1 Esterase Inhibitor
cfDNA – Cell-free DNA
CI – Confidence Interval
CitH3 – Citrullinated Histone H3
CitH3-DNA – CitH3 and DNA Colocalized Complex
CK – Creatine Kinase
CK-MB – Creatine Kinase Myocardial Band
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
CRP – C-Reactive Protein
crRNA – CRISPR RNA Array
cTnI – Cardiac Troponin I
cTnT – Cardiac Troponin T
CXCL – CXC Motif Ligand
CXCR4 – CXC-Chemokine Receptor 4
DM – Diabetes Mellitus
EMAF – European Minor Allele Frequency
FCS – Fetal Calf Serum
FXR1 – Fragile-X-Mental Retardation Related Protein 1
G-CSF – Granulocyte Colony-Stimulating Factor
GWAS – Genome-Wide Association Studies
HDR – Homology-Directed Repair
HMGB1 – High Mobility Group Protein B1
HNF4α – Hepatocyte Nuclear Factor 4 Alpha
IGV – Integrative Genomic Viewer
IQR – Interquartile Range
IRAK1 – IL-1 Receptor-Associated Kinase 1
KI – Knock-In
KO – Knock-Out
LDL – Low-Density Lipoprotein
LDLR – Low-Density Lipoprotein Receptor
lncRNA – Long non-coding RNA
LPS – Lipopolysaccharide
m^7G – 7-Methylguanosine
MACE – Major Adverse Cardiovascular Event
MAF – Minor Allele Frequency

MDR1 – Multidrug Resistance Protein1
MEM – Minimum Essential Medium
miRISC – miRNA-Induced Silencing Complex
miRNA – microRNA
MPO – Myeloperoxidase
MRE – miRNA Response Elements
mRNA – Messenger RNA
NHEJ – Non-homologous End Joining
NE – Neutrophil Elastase
NET – Neutrophil Extracellular Trap
NGS – Next Generation Sequencing
non-STEMI – Myocardial Infarction without ST-Segment Elevation
nt – Nucleotides
OD – Optical Density
OR – Odds Ratio
PAD4 – Peptidyl Arginine Deiminase-4
PAM – Protospacer Adjacent Motif
PBS – Phosphate-Buffered Saline
pegRNA – Prime Editing Guide RNA
PKC – Protein Kinase C
PMA –Phorbol-12-Myristate-13-Acetate
PMN – Polymorphonuclear Leukocytes
PRKRA – Protein Activator of the Interferon-induced Protein Kinase
PSGL-1 – P-selectin Glycoprotein Ligand-1
RAC1 – Rac Family Small GTPase 1
RISC – RNA-Induced Silencing Complex
RNase – Ribonuclease
RNP – Ribonucleoprotein
ROS – Reactive Oxygen Species
RT – Room Temperature
qRT-PCR – Quantitative Reverse Transcription-Polymerase Chain Reaction
SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2
SCAD – Spontaneous Coronary Artery Dissection
SD – Standard Deviation
sgRNA – Single Guide RNA
SLE – Systemic Lupus Erythematosus
SMC – Smooth Muscle Cell
SNP – Single Nucleotide Polymorphism
SNV – Single Nucleotide Variant
ssODN – Single-Stranded Oligodeoxynucleotide
STEMI – Myocardial Infarction with ST-Segment Elevation
TBS – Tris Buffered Saline
TF – Tissue Factor
TFPI – Tissue Factor Pathway Inhibitor
TLR – Toll-Like Receptor
TNF – Tumor Necrosis Factor
TRAF6 – TNF Receptor-Associated Protein Factor 6
TRBP – Transactivation Response RNA Binding Protein
UA – Unstable Angina
UFH – Unfractionated Heparin
WNK4 – With-No-Lysine Kinase 4
WT – Wild Type

GENERAL INTRODUCTION

1. ACUTE CORONARY SYNDROME (ACS)

1.1. ACS epidemiology

Nowadays, cardiovascular diseases, closely followed by cancer, are the most common cause of death in Europe with approximately 4 million deaths in 2021[1]. Those cardiovascular diseases include both, stable coronary disease, which typical clinical presentation is stable angina, and acute coronary syndrome (ACS), that encompasses unstable angina and acute myocardial infarction (AMI). The incidence analysis from the American Heart Association has reported that every 40 seconds an American suffers an ACS event and the annual incidence is predicted to be around 600000 new events and 200000 recurrent events[2]. Of all ACS manifestations, myocardial infarction is the major contributor to mortality rates. Among patients older than 45 years of age, 23% of females and 18% of males will die within the first year after AMI[2].

ACS became more common with older age (**Figure 1**), the average age for the first event is 65 for males and 72 for females[2]. Therefore, it is worth noting that the European population is progressively aging. In 2019, the population older than 65 years old represented 17% of the European population[1]. Elderly patients comprise approximately one-third of AMI patients and two-thirds of the deaths resulting from it[3]. Also, ACS happens around 10 years earlier in men than in women[4] and the decrease in in deaths from ACS in recent years has been bigger in men than in women. The early mortality rates are also higher in women[5]. The proportion of women suffering ACS in Europe is estimated to be around 20-30% of all ACS cases[6].

The cost of treatment for ACS is significant, in Spain it was estimated at 1 billion euros per year, mostly consumed in revascularization procedures and hospitalization. The high cost of the clinical event is more related with resource utilization than with the costs of medication, therefore the individuals with rehospitalization involve a higher cost due to more resource utilization[7]. In Europe, one-third (two-thirds in the UK) of the total hospital spend accounts for intensive care units and coronary care units, and Clopidogrel accounts for 27% of the ACS medication cost in Spain (between 50 and 20% in Europe)[8].

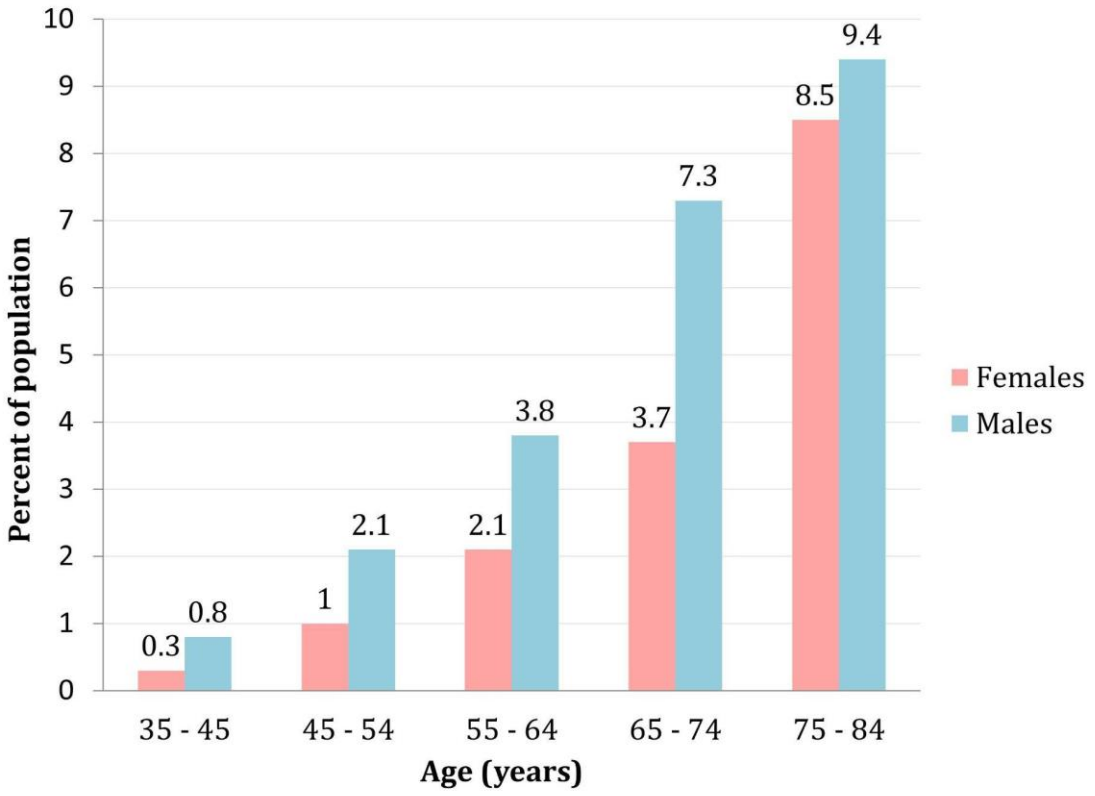


Figure 1. Prevalence of ACS between age and gender (2015 - 2018). Information from the National Health and Nutrition Examination Survey.

Depending on the cardiac electrophysiology, we can find two differentiated groups of ACS patients: Those presenting with acute chest pain and ST-segment elevation for longer than 20 minutes, this often indicate a total coronary occlusion normally leading to an ST-segment elevation myocardial infarction (STEMI); those patients with chest pain but short ST-segment elevation time, normally with cardiomyocyte necrosis leading to myocardial infarction without ST-segment elevation (non-STEMI) or, less commonly, without cell death, causing unstable angina (UA)[9].

1.2. ACS pathophysiology

The most prevalent mechanism for the appearance of ACS is the rupture of an atherosclerotic plaque, resulting in partial or complete occlusion of a coronary artery. This break exposed the subendothelial collagen, activating platelets and the coagulation cascade, leading to a thrombus. The thrombus may be partially or completely occlusive.

In the first case, patients typically have changes suggestive of ischemia that lacks the elevation of the ST-segment, namely UA or non-STEMI. When the occlusion is complete, patients generally present STEMI. The rationale in those patients is an early reperfusion with catheter-based approaches or pharmacological treatment[9,10].

The atherosclerotic plaques more prone to rupture share some anatomic characteristics and are called "vulnerable plaque". This plaque includes a thin fibrous cap, a substantial lipid core packed with inflammatory cells and a shortage in smooth muscle cells (SMC)[11,12]. Besides plaque rupture, superficial plaque erosion is also being recognized as a cause of coronary thrombosis and associated generally with non-STEMI[13]. The extent of plaque disruption (erosion, fissure or ulceration) is a crucial factor for determining thrombogenicity, actually, in the disrupted atherosclerotic plaque it can be predicted by the degree of local tissue factor (TF) activity[14].

We need also to remark the role of the endothelium in the initiation of atherothrombotic disease. Endothelium is a dynamic autocrine and paracrine organ with a highly important role in preserving vascular homeostasis, controlling vasomotion and balancing the thrombotic properties. After a mechanical or chemical signal, the endothelium responds by releasing mediators that modulate the structure and tone of the vasculature, the platelet function, monocyte adhesion and coagulation[15,16]. For instance, during hypercholesterolemia, endothelium relaxation is impaired while contraction and adhesion of monocytes and platelets are exacerbated. Endothelial dysfunction is recognized as the precursor that triggers the atherosclerotic process [14].

Atherosclerosis and inflammation

Atherosclerosis is a chronic inflammatory disease and, as described above, is by far the most prevalent pathology underlying ACS. The atherogenic process starts with the accumulation of lipoproteins in the vascular subendothelium. Specifically, the accumulation of low-density lipoprotein (LDL) correlates with the typical risk factors like smoking, hypertension or diabetes mellitus. LDL is modificated by reactive oxygen species (ROS) in the intima, promoting the uptake of oxLDL by macrophages[16,17] and triggering inflammation of the arterial wall via Toll-like receptors (TLRs), a family of pattern recognition receptors that induce proinflammatory signaling[18]. Actually, oxLDL is a clinical marker of plaque inflammation[19]. TLRs activation triggers the accumulation

of active immune cells along with the migration and proliferation of SMC, consolidating endothelial damage. The activated macrophages release chemoattractants, entering a positive feedback where more macrophages and vascular smooth muscle cells are recruited into the injured media. The ratio of smooth muscle cells and macrophages is important in determining the plaque vulnerability because of their lytic capacity. All this process leads to a cellular dysfunction in the lesion area involving cell death. To resolve it, proteases are liberated, degrading the extracellular matrix and causing the breakage of the atherosclerotic plaque[14,20].

It is well known that macrophages are a major cell type during early atherosclerosis and take part in important roles during the entire progression of the lesion (**Figure 2**), but neutrophils have been way less studied in the atherosclerotic process. In fact, neutrophils were not observed in the plaques until 2008, when van Leeuwen et al. demonstrated for the first time the presence of myeloperoxidase and neutrophils in atherosclerotic lesions from a murine model[21]. The role of neutrophils in chronic inflammatory diseases such as atherosclerosis will be broadly explained later ***(2.3. NETs and cardiovascular disease)*****.**

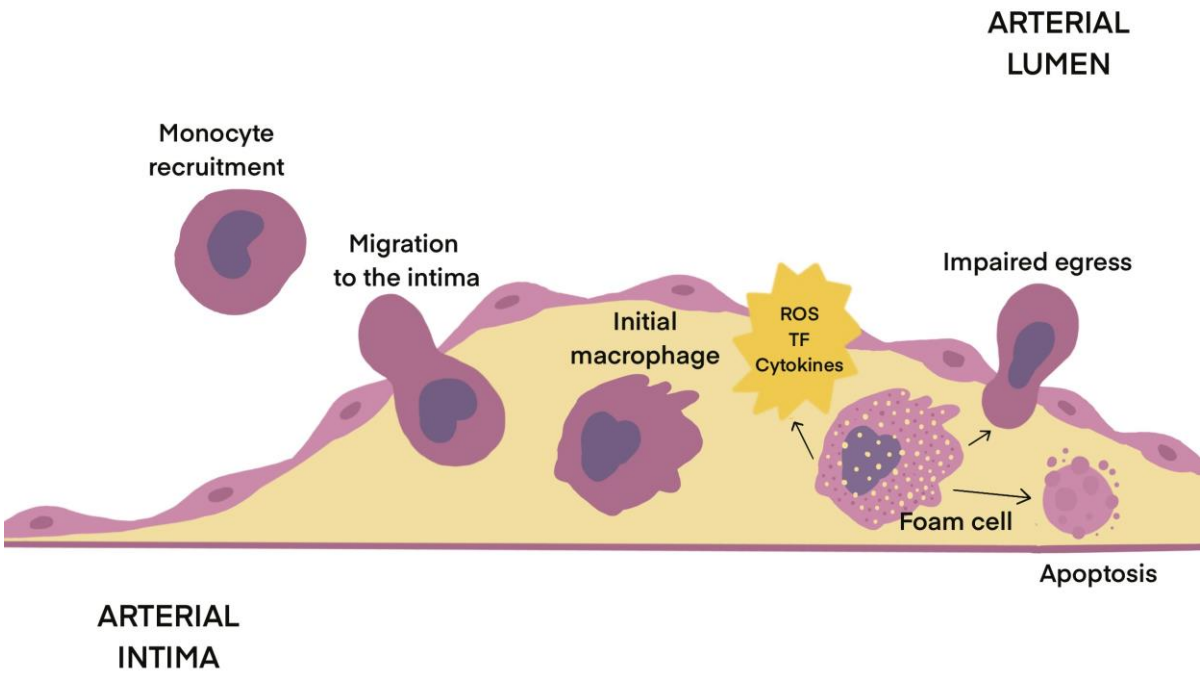


Figure 2. Schematic representation of macrophage role in atherosclerosis.

ACS in young patients

Around 6% of the ACS patients are younger than 45 years old and it is known that young ACS patients represent a unique set of patients, with their particular risk factors and clinical manifestations[22]. Hyperlipidemia and cigarette smoking have a serious role in the development of atherosclerosis at an early age as demonstrated in autopsies of 15 to 34 years old decedents[23].

A surprising characteristic of those patients is the occurrence of AMI without the presence of atherosclerosis in 20% of those cases (younger than 45 years old). Smoking habit is also different between the young and older patients. It is the most prevalent risk factor in young patients of AMI so it is conceivable that it contributes to those AMI with minimum atherosclerosis[24,25].

Another well-known risk factor in this group of ACS patients is cocaine use. The mechanisms of ACS subsequent to cocaine use are multifactorial. Cocaine enhances coronary vasoconstriction and boosts thrombosis via platelet adherence, reducing the myocardial oxygen supply[26]. The proportion of obese young ACS patients (30 to 58%) is also greater than in older patients while diabetes mellitus is more frequently associated with ACS in older than in younger patients[24].

1.3. Risk factors

Traditional cardiac risk factors are not useful for predicting ACS in patients with symptoms in the emergency department[27]. But they are useful in risk stratification, as demonstrated by several scores that include them, such as the HEART (History, Electrocardiogram, Age, Risk factors and Troponin) score[28] or the TIMI (Thrombosis In Myocardial Infarction) score[29].

Here we will focus on the risk factors of young adults (<45 years old) undergoing ACS. As the number of risk factors increases, the severity of coronary atherosclerosis also increases in those patients[30]. In the 20% of cases without atherosclerosis, the causes are similar but with some notable differences, such as recreational drug use, especially with cocaine abuse. For the young patients with atherosclerosis we found both, conventional and unusual risk factors.

Conventional risk factors

Cigarette smoking

Tabaquism is the most common risk factor in young ACS patients -specially AMI- between 80 and 90% of them are smokers, more prevalent even than male gender, while in older patients this percentage decreases to approximately 40%[31]. Furthermore, the extent of smoking is significantly related to the age of occurrence of AMI[32]. Smoking produces endothelial dysfunction and may trigger coronary spasm which may contribute to the appearance of AMI in patients without atherosclerosis[33].

Obesity

Obesity (defined as BMI > 30 kg/m^2) is also more frequent in young patients than in older ones, around 30-60% of premature patients are obese. The Framingham Heart Study showed that in patients under 50 years in the highest tertile the incidence of ACS was two times greater than in those patients in the lowest tertile[34]. High levels of fat mass are related to most cardiovascular risk factors, such as blood pressure, plasma lipids, type 2 diabetes mellitus or inflammation[35].

Dyslipidemia

In different studies of young patients, the prevalence of hyperlipidemia is reported to range from 30% to 60%[36–38] and is proposed to be a more reliable predictor of AMI in young patients, specifically in a group comprising patients between 30 and 39 years old, than in older ones[39].

Remarkably, atherosclerosis is extremely common in homozygous patients with familial hypercholesterolemia, a congenital disease characterized by its inability to remove low-density lipoprotein cholesterol from the bloodstream[40]. In addition, independently of low-density lipoprotein cholesterol or high-density lipoprotein levels, hypertriglyceridemia has been steadily identified in patients with early coronary heart disease[41].

Diabetes mellitus

As seen before, cardiometabolic factors which include obesity, dyslipidemias and diabetes mellitus (DM) plays an important role triggering the development of ACS in young patients, but DM is less common in young patients than in older ages[42], being present in only around 5% of patients younger than 45 years old, although these patients frequently have mild glucose metabolism problems[43].

Hypertension

Hypertension is also less frequent in young patients with ACS than in older patients, in whom it is the most common risk factor[42]. But still, its prevalence in young ACS is remarkable being around 30-45%[36,38].

Family history (genetics)

Up to 41% of ACS young patients have a family history of premature atherosclerotic cardiovascular disease, compared to 28% of the older patients[44]. This association is mostly due to genetics but also environmental, since normally families share and learn the same lifestyle. Even socioeconomic status has been reported to be associated with an increased risk of AMI, especially in young individuals[45].

It is known that a family history of premature death from the disease is associated with a significantly higher genetic risk of death. It has been reported, in a study of coronary angiograms from 882 siblings with AMI (from 401 families), that even the location of the lesion is markedly influenced by heritability[46]. Twin studies where both died early from ACS, have hypothesized that there must be multiple genetic factors interacting to produce death susceptibility[47].

Male gender

Some hormonal conditions, like polycystic ovary disease or pregnancy, increase the risk of infarction in females and other hormonal diseases like androgen synthesizing tumors or congenital adrenal hyperplasia, may predispose to occurrence of infarction at a young age in males and females, due to the elevated endogenous dehydroepiandrosterone sulfate levels, an independent risk factor for young but not for individuals older than 50 years of age[48].

Non-conventional risk factors

The non-conventional risk factors are those considered as non-atherosclerotic and should be evaluated in any patient with ACS younger than 45 years of age. Actually, it is important to obtain the medical history of drug use in young ACS patients. An important percentage of them are cocaine users, even the use of oral contraceptives may greatly increase the risk in those patients that are also smokers[49]. Also, psychosocial stress is decidedly underappreciated in those young patients, but the high prevalence of anxiety and depression can contribute to substance addiction and, importantly, to the pathogenesis of acute cardiovascular diseases[50–52].

Cocaine

The use of cocaine is a recognized risk factor in young patients. Cocaine increases heart rate and blood pressure and triggers vasospasms which decrease coronary blood flow giving place to increased myocardial oxygen demand and reduced oxygen delivery. The drug also induces platelet hyperaggregability and all together can lead to ischemia or infarction[53]. From a total of 10085 patients of non-fatal AMI between 18 and 45 years, 25% were frequent users of cocaine[54], another report found the average age of 130 patients presenting cocaine use associated with AMI in 38 years[55]

Coronary Artery Pathologies

Congenital coronary artery pathologies explain around 4% of AMIs in young patients[56]. Aberrant anatomy of a coronary artery amid the aorta and the pulmonary artery narrows the luminal diameter and may cause severe ischemia[57]. Spontaneous coronary artery dissection (SCAD) is also a cause of AMI particularly in young females without other risk factors. Actually, one third of all AMIs associated with pregnancy are related to SCAD[58].

Immune-mediated inflammatory diseases

Granulomatous vasculitis may affect the coronary arteries resulting in ACS. Behçet's disease may, infrequently, affect also the coronaries particularly in males with AMI. Those patients are also susceptible to early atherosclerosis[59]. The presence of antiphospholipid antibodies can also cause ACS because of the increased coagulability, they can develop in association with other disorders such as systemic lupus

erythematosus (SLE). In those patients, AMI may be the first sign of SLE and, therefore, should always be considered[60].

Infections

Acute infections can lead to ischemia and ACS by fever, hypoxia, and tachycardia, leading to an imbalance between the oxygen demand and supply in the heart[61]. On the other hand, chronic infections cause a mild chronic inflammation provoking atherosclerosis[62].

The present situation, with the COVID-19 pandemic, has increased the occurrence of young AMI. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been reported to cause both obstructive and non-obstructive coronary artery disease and SCAD. The damage of the cardiovascular system by SARS-CoV-2 is highly suspected to be related to the endothelial dysfunction and the occurrence of thromboses in those patients. There is also an hyperimmune response resulting from the cytokine storm caused by the disease as well as the release of neutrophil extracellular traps (NETs), important effectors in immunothrombosis that will be explained in the following section[63,64].

1.4. Traditional diagnostic biomarkers

A quick and precise diagnosis is crucial for the medical management of ACS patients. In that context, the measurement of cardiac biomarkers is highly useful. The onset of ACS involves the release of several factors and proteins to the blood that can be potential diagnostic biomarkers[65]. In the case of AMI, the diagnosis still depends on the electrocardiogram, the patient symptoms and the classical markers. But many patients without a significant ST-segment elevation present with nonspecific symptoms and half of them end up without the right diagnosis[66]. Classic biomarkers of cardiac necrosis such as creatine kinase or aspartate aminotransferase are poorly specific for cardiac injury due to their ubiquitous distribution, while cardiac troponins have been established as the markers of choice for ACS[67].

Cardiac Troponins

The clinical utility of cardiac troponin T (cTnT) and cardiac troponin I (cTnI) has been well established to be the most specific and sensitive diagnostic biomarkers for ACS and is considered the gold-standard for AMI[68,69].

The troponin complex acts binding Ca^{2+} and initiating the myocyte contraction, resulting in the increased mobility of tropomyosin over the actin strands. The complex has three subunits: cTnC, cTnI and cTnT. cTnC binds to Ca^{2+} to produce movement, cTnT binds to tropomyosin, becoming troponin-tropomyosin complex, and cTnI binds to actin to retain the troponin-tropomyosin complex[70]. The complex is only produced in the myocardium, but cTnC is not useful as a cardiac biomarker, while the other two subunits are only detected in blood when there is heart injury, and false positives are pretty rare[71]. The cardiac troponins take up to 10h after symptoms to appear in serum, the levels peak at 12-48 hours and they have a large temporal window, they remain elevated for 4 to 10 days after the onset of AMI[72]. Something to keep in mind nowadays is that many patients undergoing COVID-19 present with elevated levels of cardiac troponins, which can be confusing for clinical interpretation. These patients present stable cTnT while ACS patients present changing values of this marker[73].

Creatine kinase and creatine kinase-MB

Before the apparition of cTnT and cTnI, creatine kinase (CK) and creatine kinase-myocardial band (CK-MB) were the gold-standard for AMI diagnosis. CK-MB is an isoenzyme of CK, mostly found in the myocardium. Similarly to cTnT and cTnI, their elevation starts around 4-6 hours after the onset of damage, but it remains only for 24-48 hours. Thus, it is a sensitive marker, but its specificity is ruined by the ubiety of this marker in skeletal muscle. To alleviate this problem in specificity, the ratio CK-MB / total CK is used and ratios greater than 2.5 are suggestive of myocardial damage[74,75]. In clinical practice, CK-MB is no longer considered as effective for detecting damaged myocardial tissue, but the area under the release time curve of CK-MB from serial samplings can be used to estimate the infarct size[76] and it remains valuable in the diagnosis of AMI[69].

B-type natriuretic peptide

B-type natriuretic peptide (BNP) is normally secreted in the left ventricle but, in response to disease, the right side of the heart can also secrete it. The stimulus controlling this release is wall stretch, but other stimuli that are activated in heart failure can also trigger it such as norepinephrine, glucocorticoids, and proinflammatory cytokines. Even despite the fact that BNP is also elevated in diseases that involve an increased fluid volume, such as renal failure or ascitic cirrhosis, plasma BNP is considered a reliable marker of left ventricular hypertrophy. It was also proposed to be useful in the evaluation of the antihypertensive therapy in patients with arterial hypertension, indicating the reduction of left ventricular hypertrophy, or as a prognostic marker for long-term survival after AMI[77]. But it is considered inadequate for the diagnosis of AMI[69].

C-Reactive protein

The most intensively studied marker of inflammation is C-Reactive protein (CRP), it is synthesized by the liver after the stimulation with cytokines (especially IL-6, IL-1β, and tumor necrosis factor (TNF)-α) in response to infection or injury[78] and its half-life is 19 hours. The direct association among elevated levels of CRP in serum and the risk of cardiovascular disease has been widely demonstrated, there is also a strong association between the concentration of CRP on admission for AMI and the long-term outcome[79], but CRP is used nowadays for monitoring inflammatory process and the course of coronary artery disease or other coronary artery pathologies, but not for the diagnosis of ACS[69].

Actually, CRP plays an active role in the innate immune response activating the complement system and regulating the expression of the endothelial NO synthase, triggering the activation of the NF-κB and the molecules of adhesion and, even, directly controlling the oxidation of the LDLs[80].

Patients presenting with UA and elevated concentrations of CRP in plasma had a higher rate of AMI, need for revascularization or even death compared with patients with normal levels and that prognostic power is notably incremented when combined with Troponins[81,82]. Remarkably, CRP has a prognostic value even in those patients with negative cTnT/cTnI and no evidence of myocyte necrosis[83].

Proposed guidelines

The role of CRP in comparison to troponins and BNP in ACS has been widely studied and discussed. The most recent guides from the American College of Cardiology (ACC) and the American Heart Association (AHA)[84] dictate that the most important role of biomarkers in clinical practice for chest pain symptoms is the fast identification or exclusion of myocardial injury. For that, the gold-standard biomarkers are cTnT or cTnI due to their high specificity and sensitivity in myocardial tissue. Even so, these markers are not disease-specific, diverse ischemic causes of cardiomyocyte injury can elevate cTnT and cTnI levels, therefore, the interpretation of these markers must be integrated with all the clinical information[84].

1.5. Anticoagulant and antiplatelet treatment

The management of ACS has substantially evolved over the last decades due to the amount of knowledge collected from basic research and clinical trials[85]. When treating younger patients the treatment must be determined taking into account the principal cause of the ACS.

In the event of ACS related to the traditional risk factors, due to plaque rupture, the medical management should be directed by the current guideline both in young and older patients. **Dual antiplatelet therapy** should be initiated as soon as possible, Aspirin (acetylsalicylic acid or ASA) and a parenteral anticoagulant, normally unfractionated heparin (UFH), are the first choice in all patients. In those patients with STEMI, a $P2Y_{12}$ inhibitor (prasugrel or ticagrelor), should be combined with the ASA and UFH and, for the patients without ST-segment elevation, a platelet GPIIb/IIIa receptor antagonist should be administered in case of continuing ischemia or any other high risk-features. For the patients unable to take ASA, a thienopyridine (clopidogrel or ticlopidine) should be administered[86,87]. In other scenarios, such as recreational drug use, which needs to be considered in every young patient, especially in patients without traditional cardiovascular factors, once detected, those patients should undergo special counseling and treatment[88].

High platelet reactivity to adenosine diphosphate (ADP) during the treatment with a $P2Y_{12}$ inhibitor is associated with ischemic events, on the opposite side, low platelet reactivity to ADP during treatment is associated with risk of bleeding. The routinary platelet function testing is not recommended, but may be considered in

patients at high risk and the concept of a therapeutic window for personalized antiplatelet therapy may improve the balance between safety and efficacy[89] (**Figure 3**). The duration of dual antiplatelet therapy in ACS patients was evaluated by the ACC/AHA in 2016. In patients who have tolerated the dual antiplatelet therapy without bleeding and that are not at risk of bleeding, the recommendation was dual antiplatelet therapy for longer than 12 months[90]. It is clear that antithrombotic and hemorrhagic responses to the treatment are not uniform and a more personalized procedure will reduce further recurrent events.

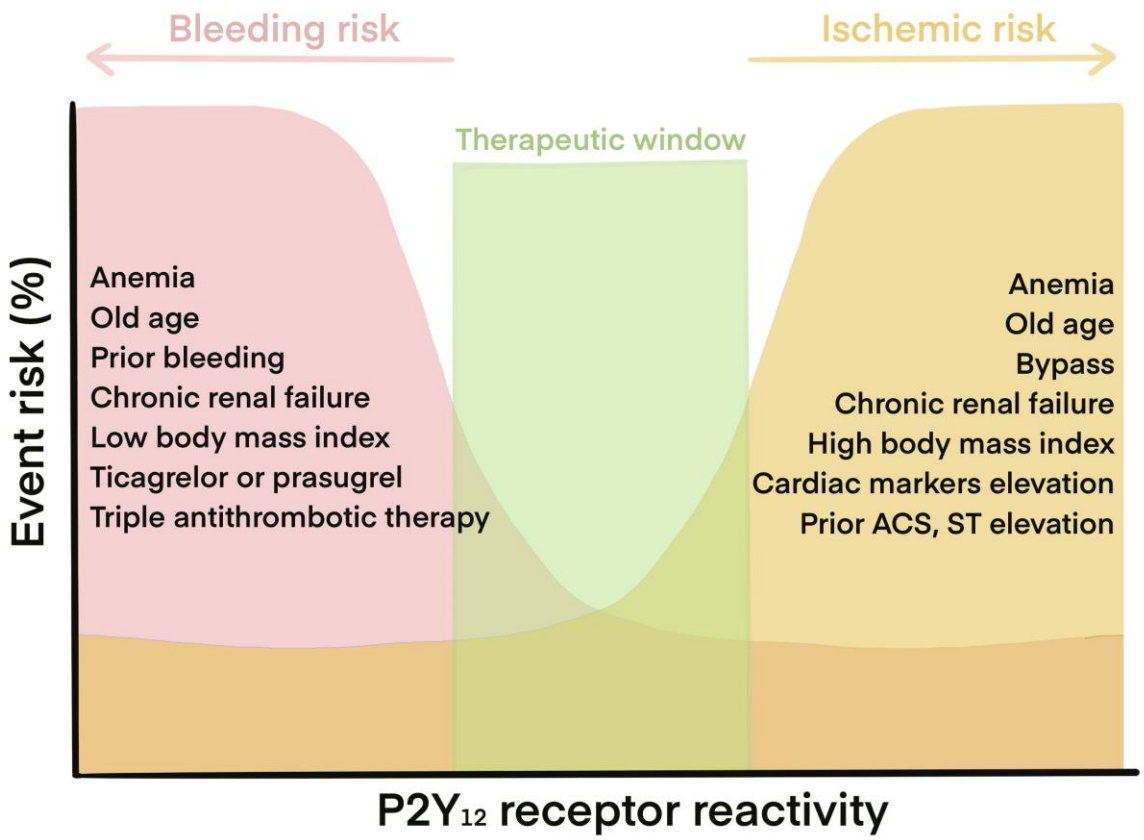


Figure 3. Therapeutic window for the personalized antiplatelet therapy. Influence of demographic variables along with platelet reactivity to ADP is associated with the risk of bleeding or ischemia and, therefore, can delimitate the therapeutic window for the treatment of those patients.

2. NEUTROPHIL EXTRACELLULAR TRAPS (NETs)

2.1. Neutrophils

Neutrophils are the most abundant leukocytes in human blood, representing around 60-70% of them. They are polymorphonuclear leukocytes (PMNs) and are the first to be recruited to an inflammatory site in defense from pathogens. Actually, the recruitment of neutrophils in the inflammation site is crucial for the clearance of the infection[91]. In physiological conditions, they can be found in the lung, spleen, liver, and bone marrow (BM), but they can move rapidly to sites of inflammation or infection[92]. They are also cleared from the circulation mainly in the spleen, liver, and BM[93]. It is known that aged neutrophils have increased CXC-chemokine receptor 4 (CXCR4), which helps direct them back to the bone marrow where they are eliminated. It can also negatively regulate the release of new neutrophils from the BM[94]. They can also die in the vasculature where Kupffer cells will remove them. Remarkably, in 2004 our understanding of neutrophil death changed with the first report of NETosis. It is a process where neutrophils break and release their nuclear content as NETs. These structures are formed by a scaffold of DNA covered with histones, antimicrobial proteins such as myeloperoxidase (MPO), neutrophil elastase (NE), lactoferrin, and cathepsin G among others, able to lock up pathogens and eliminate them[95,96] (**Figure 4**).

2.2. NET formation mechanisms

NETs were identified for the first time as a defense mechanism, thus mice unable to produce NETs have a high susceptibility to invading pathogens, proving the importance of this process during infection[97]. The NET formation process is called "NETosis", and it was firstly described as a cell death process different from apoptosis or necrosis. But, in some cases, the stimulation of neutrophils can induce a form of NETosis, called vital NETosis, in which the neutrophil is still able to perform phagocytic functions after releasing NETs. Therefore, NETosis can end with cell death or with vital NET release (**Figure 5**). Of note, it seems that the most important part determining the kind of NETosis is the stimulus. Stimulation with a chemical molecule, phorbol-12-myristate-13-acetate (PMA), led to the discovery for the first time of the production of NETs[98,99].

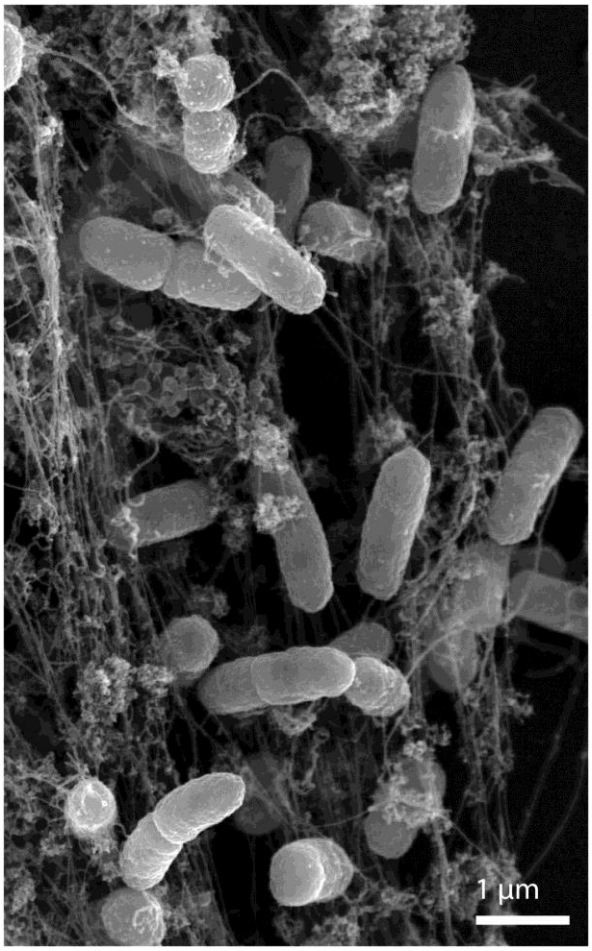


Figure 4. Image of NETs obtained by electron microscopy. The image presents human NETs trapping *Salmonella*. (*Figure from Brinkmann et al., 2004, Science)* [95]

Suicidal NETosis

PMA, autoantibodies or cholesterol crystals can induce suicidal NETosis. It all begins with the activation of NADPH oxidase, then ROS are produced and the peptidyl arginine deiminase-4 (PAD4) is activated[96,100]. We know that PAD4 is indispensable in the process of NETosis since *Pad4*$^{-/-}$ mice are unable to form NETs. PAD4 is responsible for the hypercitrullination of histones, triggering decondensation of chromatin necessary to form NETs[96,101]. After the decondensation of chromatin mediated by PAD4, NE, and MPO are translocated to the nucleus triggering even more the unfolding of chromatin and, therefore, the disruption of the nuclear membrane with the liberation of the chromatin in the cytosol, there the DNA get covered by granular and cytosolic proteins. Finally, the plasma membrane breaks, the NET is liberated and the neutrophil dies. Suicidal NETosis requires hours of stimulation[100] (**Figure 5**).

Vital NETosis

Vital NETosis takes minutes to happen after stimulation of neutrophils with bacteria or their products, activated platelets (via TLR4) or complement proteins with TLR2 ligands[94,102]. Another main difference with suicidal NETosis is that it can occur idependently of ROS and the plasma membrane is untouched, since NETs are released via vesicular export. After stimulation, PAD4 is activated inducing chromatin decondensation, the rest of the process remains the same as suicidal NETosis, except for the breakage of the plasma membrane. It is called vital NETosis because some authors have proposed that neutrophils can still exert their functions after undergoing it[103,104] (**Figure 5**).

Molecular mechanisms regulating NETosis

Some other stimuli, like ionomycin, immune complexes or nicotine, can trigger NETosis separately of NADPH oxidase, employing only mitochondrial ROS[105]. On the other hand, in pulmonary fungal infection, the release of NETs is abolished in NADPH oxidase-deficient mice[106].

Some ROS, such as hydrogen peroxide, trigger the selective release of NE into the cytosol with dependence of MPO, which activates the proteolytic activity of NE. However, the inhibition of MPO activity does not block NETosis, but delays it[107,108]. Once activated, NE binds to F-actin filaments and degrades them to enter the nucleus[100] and it is known that NE can decondense the nucleus *in vitro*[108]. Another protein implicated in NET formation is DEK, a nuclear chromatin-binding protein. NETosis is abrogated in *Dek*-deficient neutrophils, since DEK promotes chromatin decondensation, and it is rescued with the addition of exogenous recombinant DEK protein[109].

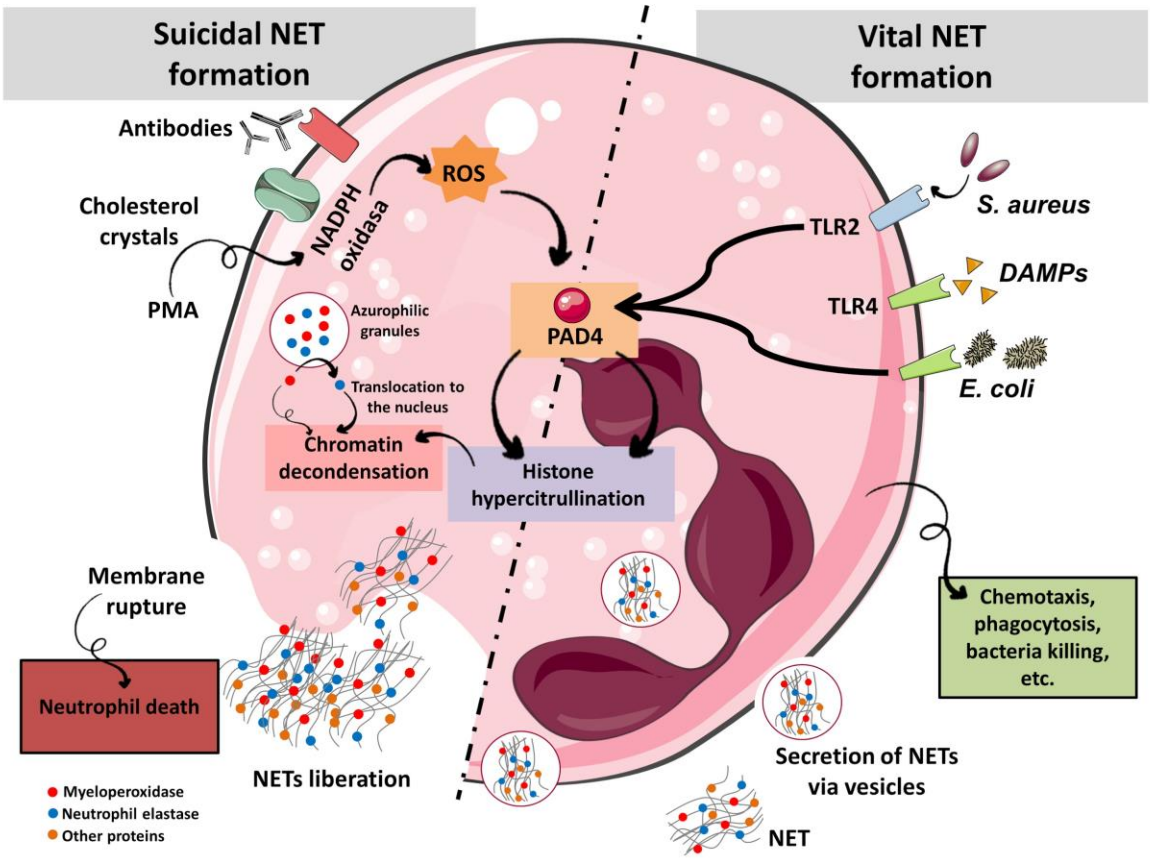


Figure 5. Overview of the different types of NETosis, suicidal or vital. (*Figure published in International Journal of Molecular Sciences in 2021)*[104]

Remarkably, ROS not only activates chromatin decondensation, it can also crosslink NET proteins therefore increasing NET stability and integrity and enhancing the capture of microorganisms[110]. As shown, there are several different pathways implicated in the generation of NETs[111] (**Figure 6**).

As briefly mentioned before, another essential modification in NETosis is chromatin decondensation by histone deamination or citrullination, driven by PAD4. This enzyme acts citrullinating arginine residues and converting amine groups to ketones[112]. Inhibition of NADPH oxidase reduces citrullination and hydrogen peroxide is enough to activate PAD4 which is activated by protein kinase C (PKC), implicated in the ROS burst[113,114]. Therefore, PAD4 is known to be downstream of ROS signaling during NET formation. Notably, PAD4 deficiency blocks NETosis induced by lipopolysaccharide (LPS), TNF, and nicotine, but does not affect NETosis induced by cholesterol crystals[115,116].

In response to several stimuli (PMA, microorganisms, parasites, etc.), ROS-inducing receptors and kinases are linked to NET formation (**Figure 6**). The phosphoinositide 3-kinase (PI3K) links NETosis and autophagy, since both require it, and LC3B vacuoles, autophagosomes, have been observed in neutrophils during NETosis. Also, ROS induce autophagy in a positive loop where autophagy is needed to maintain ROS burst[117].

Although NETs are crucial for human host defense, it may be detrimental. Many of the antibacterial properties of NETs are dependent on their procoagulant function. An exacerbated production of NETs or a failure in their clearance can bring detrimental consequences, mostly related to thrombosis. Therefore, NETs play a crucial role in immunothrombosis.

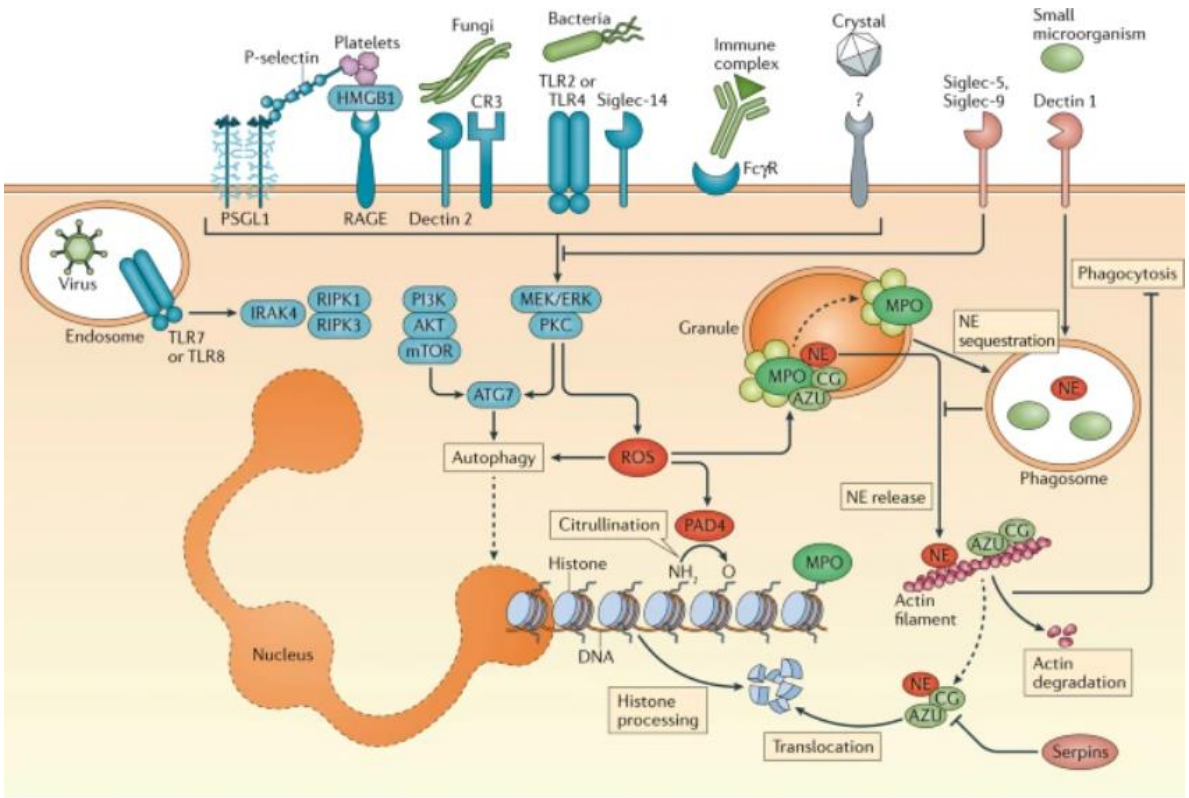


Figure 6. NETosis is triggered by several stimuli, both endogenous or external, such as immune complexes, bacteria, fungi, etc. Each stimulus can activate specific receptors and, therefore, different downstream pathways, all giving place to NET formation. *(Figure from Papayannopoulos, 2018, Nature Reviews Immunology)*[118]

2.3. Immunothrombosis

In 2012, Engelmann and Massberg coined the term "immunothrombosis", and defined it as "consequence of the evolutionary conserved link between blood coagulation and innate immunity". It is a physiological process of intravascular immunity that comprises a wide variety of host strategies to protect against pathogens. But, when immunothrombosis is dysregulated due to intense stimuli or due to failure of its control mechanisms, it will probably lead to thromboinflammation, involving the development of thrombotic disorders[119].

During arterial thrombosis induced by atherosclerotic plaque rupture, the recruitment of leukocytes takes place under high-shear conditions and is backed by platelets. Similarly to physiological haemostasis, platelets are recruited to the exposed subendothelium, they get activated and, therefore, express adhesion receptors such as P-selectin and release chemokines that induce the recruitment of innate immune cells[120]. At the time of systemic infections, the simultaneous activation of coagulation and inflammation represents a pathological loop where immune cells are excessively activated and an intravascular thrombus is formed, therefore this process becomes highly detrimental for the host tissue[119].

As discussed before, neutrophils have also specific mechanisms that can trigger thrombosis such as NETs. Beside the antibacterial function, NETs also induce a potent procoagulant response, mainly by binding and activating platelets. Moreover, the nucleosomes that compose the NETs form a catalytic platform that potentiates the proteolytic activity of NE that induces the inactivation and degradation of tissue factor pathway inhibitor (TFPI). NETs can even capture TF and extracellular vesicles carrying TF and, consequently, trigger the TF-induced coagulation[121].

One of the first described physiological stimuli to induce NETs were activated platelets. In bacterial sepsis, LPS triggers activation of platelets without P-selectin exposure, and NETs are formatted due to the interaction of neutrophils with these activated platelets through TLR4[122]. In sterile conditions, platelets expose P-selectin that interact with P-selectin glycoprotein ligand-1 (PSGL-1) of the neutrophils to trigger NET formation[123]. Platelet high mobility group protein B1 (HMGB1) also causes neutrophil recruitment and activation[124] and, therefore, NET formation, as it will be discussed later (CHAPTER 2) (**Figure 6**).

NET components leading to immunothrombosis

The components of NETs that lead to immunothrombosis are a new class of damage-associated molecular pattern molecules (DAMPs), molecules released from dying or damaged cells that can activate the innate immunity via pattern recognition receptors (PRRs)[125]. The presence of **cell-free DNA (cfDNA)** in human plasma has been well known since the 1950s and is an indicator of the immune response in diverse diseases. The excessive cfDNA in circulation was supposed to come from an increased cell death or activation. But a deficient clearance of cfDNA may also be due to the decrease of endogenous DNAse activity [126]. It is known that an important part of this cfDNA, during proinflammatory responses, is due to NETosis[95,127]. DNA and RNA are polyanionic molecules that can trigger the activation of proteases of the contact pathway such as FXII and XI[128]. Also, this polyanionic surface can enhance the capacity of NE to inactivate TFPI[129]. cfDNA at physiological concentrations can induce the activation of plasminogen by the tissue-type plasminogen activator (tPA), increasing fibrinolysis. On the other hand, it can also suppress fibrinolysis via inactivation of tPA at higher concentrations[130]. *In vitro*, the digestion of cfDNA by DNase I can abolish the procoagulant effects. It can dismantle the scaffold formed by DNA, release histones, and remove platelet aggregates from the NETs[131].

Another DAMP released with NETs are **extracellular histones**. Their cytotoxicity for endothelial cells has been demonstrated *in vitro* and *in vivo* in murine models of LPS induced sepsis or cecal ligation and puncture sepsis[132]. Also, on human alveolar epithelial cells, the treatment with anti-histone antibodies managed to decrease the cytotoxic effects of NETs[133]. Histones are able to signal *in vitro* via TLR2 and TLR4 and have a role as NETosis inducers[134,135]. In summary, the release of histones by cell death induces immune cell recruitment via PRRs, triggers NET formation and, therefore, leads to the active release of more histones, all of it promoting local damage. Finally, as described above, some neutrophil enzymes such as NE and cathepsin G also act like DAMPs, leading to immunothrombosis.

Remarkably, it has been demonstrated that purified DNA or recombinant human histones H3 and H4 can trigger thrombin generation but intact NETs are unable, as well as nucleosome particles or octameric core histones *in vitro*. The proposed explanation is the neutralization of the negative charges in the histone-histone or histone-DNA complexes and the difference in binding properties with individual histones[136].

2.4. NETs in acute coronary syndrome

In the past, the inadequate methods available to detect PMNs and their limited lifespan have driven investigators to underestimate the contribution of neutrophils in **atherosclerosis**, but their role is quite important during this inflammatory process. Hyperlipidemia induces neutrophilia which level is positively correlated with the extent of lesion formation[137]. Also, hypercholesterolemia induces the elevated production of granulocyte colony-stimulating factor (G-CSF) which reduces CXC motif ligand (CXCL)-12, which ends in the reduction of the clearance of aged PMNs, and boosts the levels of CXCL-1, promoting PMN mobilization[138]. Those increased PMNs attach to atherosclerotic plaques through NETs, and their components also take part in the atherosclerotic process. Cathepsin G attracts monocytes in atherosclerotic plaques and MPO induces ROS and pro-inflammatory cytokines release. These NET components also affect plaque stability and the ROS liberated modify LDL to ox-LDL, leading to foam cells[140–143]. Also, the cathelicidin-related antimicrobial peptide (CRAMP) liberated from neutrophil secondary granules can recruit and activate dendritic cells[144] and, in combination with the cfDNA, lead to a substantial liberation of type I interferon by them, affecting atherosclerotic plaque burden[145,146] (**Figure 7**).

In 2013 when Borisoff et al. studied for the first time the plasma levels of cfDNA, nucleosomal fragments, and NET formation in cardiovascular disease[139]. Megens et al. reported for the first time the presence of NETs in atherosclerotic lesions from humans and mice[147]. Actually, they were found near apoptotic endothelial cells and SMC, remarking their importance for plaque destabilization. This importance is further confirmed by the research showing that inhibition of PAD4 produces a decrease in atherosclerotic lesion size and postpones carotid artery thrombosis in mice therefore, targeting PAD4 may have a therapeutic potential in treatment and prevention of atherosclerosis[148,149].

Atherothrombosis is the formation of a thrombus in an atherosclerotic artery. Circulating leukocytes, especially monocytes, have an important role in this disease and neutrophil counts are powerful predictors of the risk of cardiovascular diseases[150]. Thrombus aspirations from AMI patients contain plentiful activated neutrophils[151] . In the site of atherothrombosis, rupture of atherosclerotic plaques provoques the aggregation of platelets and fibrin deposition. These platelets interact with neutrophils during STEMI triggering NETosis[121]. It has been described, in coronary thrombectomies from 111 STEMI patients, that NET burden and DNase I activity predict myocardial infarct size and ST-segment resolution[152]. NETs covered with TF were also detected in thrombi from infarcted tissues, the NET formation during AMI could trigger the delivery of activated TF, which can foster thrombus formation[121,153].

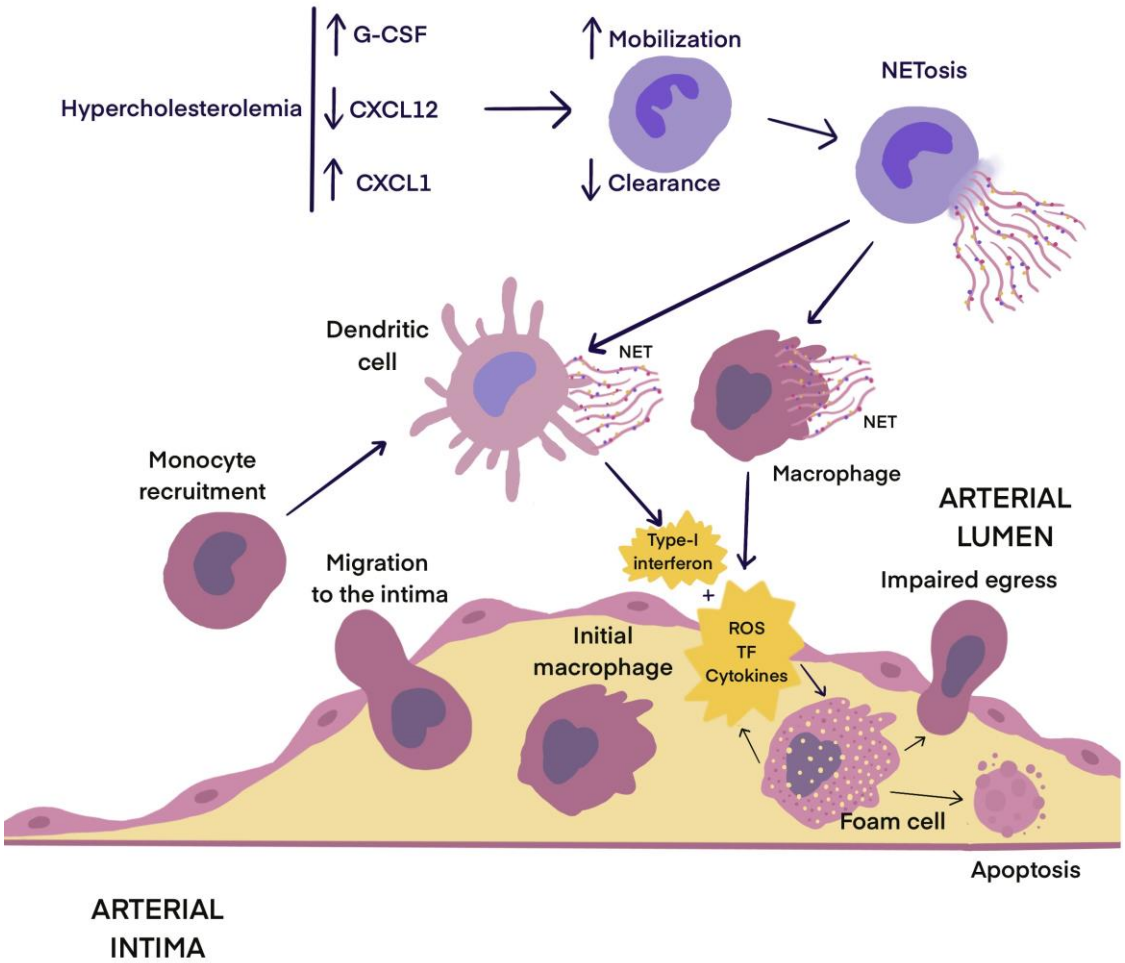


Figure 7. Schematic representation of neutrophils and NETs role in atherosclerosis. A. ROS: Reactive oxygen species. TF: Tissue Factor. G-CSF: Granulocyte colony-stimulating factor. CXCL: CXC motif ligand.

2.5. NET clearance

Clearance of NETs happens in two-steps; first, the large structure that forms the backbone of the NET is fragmented in smaller structures, then these NET fragments should be removed by phagocytic cells. Since the backbone of NETs is mainly DNA, endogenous DNases can dissolve them, as it was proven in the first study that reports NET existence[98] and afterwards, widely confirmed by many other studies[154,155]. It is worth noting that DNase I is already approved to treat cystic fibrosis, via inhalation of an aerosol (recombinant human DNase I), since this disease is typically accompanied by a high NET production in lungs. This approach was proposed in the 1990s, long before the discovery of NETs[156]. An *in vivo* study identified the existence of two active DNases in sera from mice. DNase I, which cleaves free DNA and is secreted by non-hematopoietic tissues, and DNase1-like 3 protein (DNase1L3), which cleaves DNA forming complexes with protein and is secreted mostly by immune cells. This study showed that the presence of at least one of these two DNases is necessary to prevent the death of the animal in a mice model lacking the DNA-degrading activity in serum[157]. Additionally, DNase I is responsible for NET digestion in serum collected from SLE and healthy individuals and a genetic deficiency of any of them induce spontaneous SLE[158–160].

By means of intravital microscopy we know that, *in vivo*, injected DNase I dissolves DNA of NETs very efficiently, but other components of NETs persist attached to the vessels due to their adherence to the glycocalyx covering the endothelium given that this glycoproteins are strongly negatively charged and histones or elastase are positively charged[161]. These remaining components can still cause collateral damage. But, in that context, our knowledge is much more vague, the first two works in this matter led to the conclusion that macrophages can phagocyte NETs[162,163]. Both reports were performed using monocyte-derived macrophages either adding them to NETotic neutrophils, or incubating isolated NETs with them. In both, NE was reported by immunocytochemistry inside macrophages. The most recent information suggests that macrophages need to be primed to digest NETs, however all macrophages activated with DAMPs or PMA have the pro-inflammatory phenotype, M1[164,165]. Notably, monocyte-derived macrophages are capable of engulfing NETs without addition of exogenous DNases, but the process is significantly facilitated when DNase I is added to the media[163].

2.6. NET-targeting therapies

It is difficult to find an appropriate treatment to reduce NETs or avoid their formation, since their function is mostly physiological and necessary to fight infections. Nowadays, the only NET-targeting therapy in clinical use is DNase, as mentioned before, but some preclinical studies show promising results.

As described before, **DNase I** is already applied to treat cystic fibrosis[156] and it has also been used to treat virus-associated bronchiolitis[166]. In SARS-CoV 2, NETs also cause mucus accumulation and airway occlusion and rigidity, therefore, a pilot clinical study investigated the effect of this Dornase alfa aerosol on COVID-19, it has only been tested in 10 patients but there was a positive effect on oxygenation accompanied with a decrease of MPO-DNA complexes (specific marker of NETs) in the lungs. Notably, this decrease of NETs did not correlate with an increase in secondary infections[167].

As discussed before, disentangling NETs may release histones and proteases with cytotoxic consequences and different mice models have demonstrated that neutralization of **histones** can be a promising therapy target. A serine protease inhibitor able to bind and neutralize histones is the C1 esterase inhibitor (C1INH)[168]. This ability is due to its negative charge produced by glycosylation. The treatment with C1INH has already been shown to produce a decrease in neutrophil activation and an improvement in inflammation and survival in sepsis[169,170]. In mice, neutralizing the citrullinated histone H3 (citH3) with an anti-citH3 monoclonal antibody limited the endothelial damage *in vitro* and improved inflammatory responses and survival *in vivo* in LPS-induced sepsis[171]. Even the heparin (a glycosaminoglycan with anticoagulant properties) is able to antagonize the effect of histones and has been demonstrated to decrease organ damage and death in rats[172].

Another promising therapy target is **HMGB1**, since the antidiabetic drug metformin directly binds HMGB1 and has been shown to decrease NET components in plasma in pre-diabetes patients independently from glucose control[173,174]. **Aspirin** treatment also decreases NETosis in lung and plasma, but did not show a significant improvement in a clinical study with low-dose aspirin in sepsis patients[175]. In a murine model of pancreatic adenocarcinoma, **hydroxychloroquine** was able to inhibit NETs and reduce hypercoagulability, however results in SARS-CoV-2 patients did not support the use of this treatment[176–178]. A summary of those promising NET-targeting therapies is listed in **Table 1.**

Table 1. Summary of NET-inhibitor treatments with clinical or preclinical application.

TARGET	TREATMENT	DISEASE MODEL	REFERENCES
DNA	Dornase Alfa (DNase)	Cystic fibrosis	Shak, 1995 [156]
		Bronchiolitis	Nasr, 2001 [166]
		SARS-CoV-2	Holliday, 2021 [167]
Histones	C1INH	Sepsis	Caliezi, 2000 [168] Igonin, 2012 [170]
	Heparin	Organ dysfunction	Iba, 2015 [172]
Citrullinated Histones	anti-citH3 antibody	Sepsis	Deng, 2019 [171]
HMGB1	Metformin	Acute liver injury	Horiuchi, 2017 [173]
		Type-2 diabetes	Menegazzo, 2018 [174]
NET formation	Aspirin	Sepsis	Eisen, 2021 [175]
	Hydroxychloroquine	Pancreatic cancer	Boone, 2018 [176]
		SARS-CoV-2	Molina, 2020 [177] Mahevas 2020 [178]

3. MICRORNAs (miRNAs)

3.1. non-coding RNAs and miRNAs history

When looking at different species, we can rank them based on complexity of the organism and find a correlation between complexity and the amount of non-coding genome. Interestingly, in humans 98% of the genome is non-coding[179] (**Figure 8**). But being non-coding does not imply that it is not active, in fact, 80% of this non-coding genome is transcribed into some type of molecule. Among them, we differentiate between the long non-coding RNAs and the small non-coding RNAs. Long non-coding RNAs (lncRNAs) are the part of the non-coding genome longer than 200 nucleotides and they stand out because they are spliced like messenger RNAs (mRNAs). On the other hand, small non-coding RNAs are transcripts smaller than 200 nucleotides and there are

several types such as small interfering RNAs, ribosomal RNAs, etc[180]. Here, we will focus on microRNAs (miRNAs).

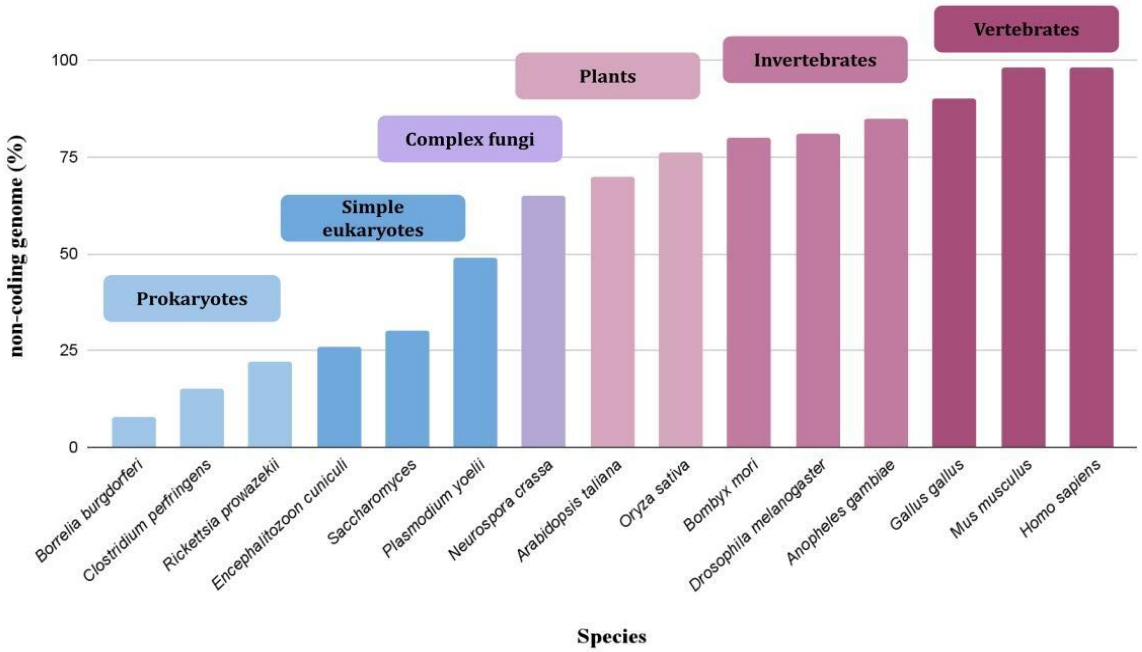


Figure 8. Correlation between complexity of the species and the percentage of non-coding genome.

The miRNAs were described for the first time by Rosalind Lee and Victor Ambros in 1993 when studying *C. elegans.* They found a small RNA called lin-4 that was able to negatively regulate the levels of the protein called Lin-14[181]. For the next 7 years, it was thought that this small regulatory RNAs were a unique phenomenon in nematodes, until the year 2000, when the presence of miRNAs in several other species was discovered, including humans, this highlighted the importance of these molecules and raised the suspicion that this non-coding RNAs were part of a larger phenomenon[182]. In 2002, Calin et al. demonstrated for the first time the correlation between human diseases and the presence and abundance of miRNAs, specifically miR-15 and miR-16 in chronic lymphocytic leukemia[183]. This discovery marked a turning point in the importance of miRNAs, constituting since then an area of study in constant growth. Six years later, Mitchell et al. demonstrated the presence of miRNAs in plasma and their stability against endogenous RNase activity. Even exposed to extreme conditions, miRNAs remain intact and quantifiable in biofluids, therefore making them a very interesting non-invasive biomarker[184].

3.2. Biogenesis of miRNAs

Around half of all identified miRNAs are intragenic and processed from introns of proteins coding genes. Only a few are processed from exons of protein coding genes. The other half are intergenic, regulated by their own promoters[178]. There are two types of miRNA biogenesis, the canonical and the non-canonical pathways.

The canonical pathway of miRNA biogenesis

It is the main pathway of miRNA processing. A pri-miRNA transcript is generated in the nucleus by RNA polymerase II, resulting in long transcripts with hairpin structures. Afterwards, they are processed into long pre-miRNA, around 100 nucleotides, by a ribonuclease (RNase) III enzyme called Drosha. Drosha cleaves the pri-miRNA duplex at the base of the hairpin structure[185]. Then, the pre-miRNA is exported to the cytoplasm by an exportin 5-mediated mechanism[186]. In the cytoplasm, pre-miRNA bears an additional step via a complex formed by the RNase III Dicer, the protein activator of the interferon-induced protein kinase (PRKRA) and the transactivation response RNA binding protein (TRBP). This step results in a ∼22 nt RNA duplex that includes the mature -5p miRNA guide and the complementary strand, -3p[187]. In the next step, one strand is selected as the guide strand (sometimes both strands) and included in the RNA-induced silencing complex (RISC). The selection of the -5p or -3p strand as the guide is based on the thermodynamic features of each end[188]. The strand used determines the name of the mature miRNA. Then, the mature miRNA duplex can be loaded into a family of proteins called Argonaute (AGO) in an ATP-dependent process[189] becoming then the miRNA-induced silencing complex (miRISC) formed by the strand guide and AGO. The complementarity degree between the miRNA and the target mRNA determines the next step, or slicing of the mRNA by AGO2 or the miRISC-mediated inhibition of the translation and mRNA decay[190,191]. (**Figure 9**)

The non-canonical pathway of miRNA biogenesis

Nowadays, multiple biogenesis pathways different from the canonical one have been elucidated, they use different combinations of proteins that are also involved in the canonical pathway. They can be grouped as Dicer-independent or Drosha-independent pathways[192]. **Dicer-independent** miRNAs are processed from endogenous short hairpin RNA transcripts by Drosha and require AGO2 because they are too small to be Dicer-substrates[193]. On the other hand, the **Drosha-independent** pathway produces pre-miRNAs that are substrate for Dicer, an example are the mirtrons[194,195]. Another example is the 7-methylguanosine-capped pre-miRNA, exported into the cytoplasm by exportin 1 without Drosha cleavage. They generate a strong bias toward the -3p strand due to the m^7G-cap preventing the -5p strand to be loaded into AGO[196]. (**Figure 9**)

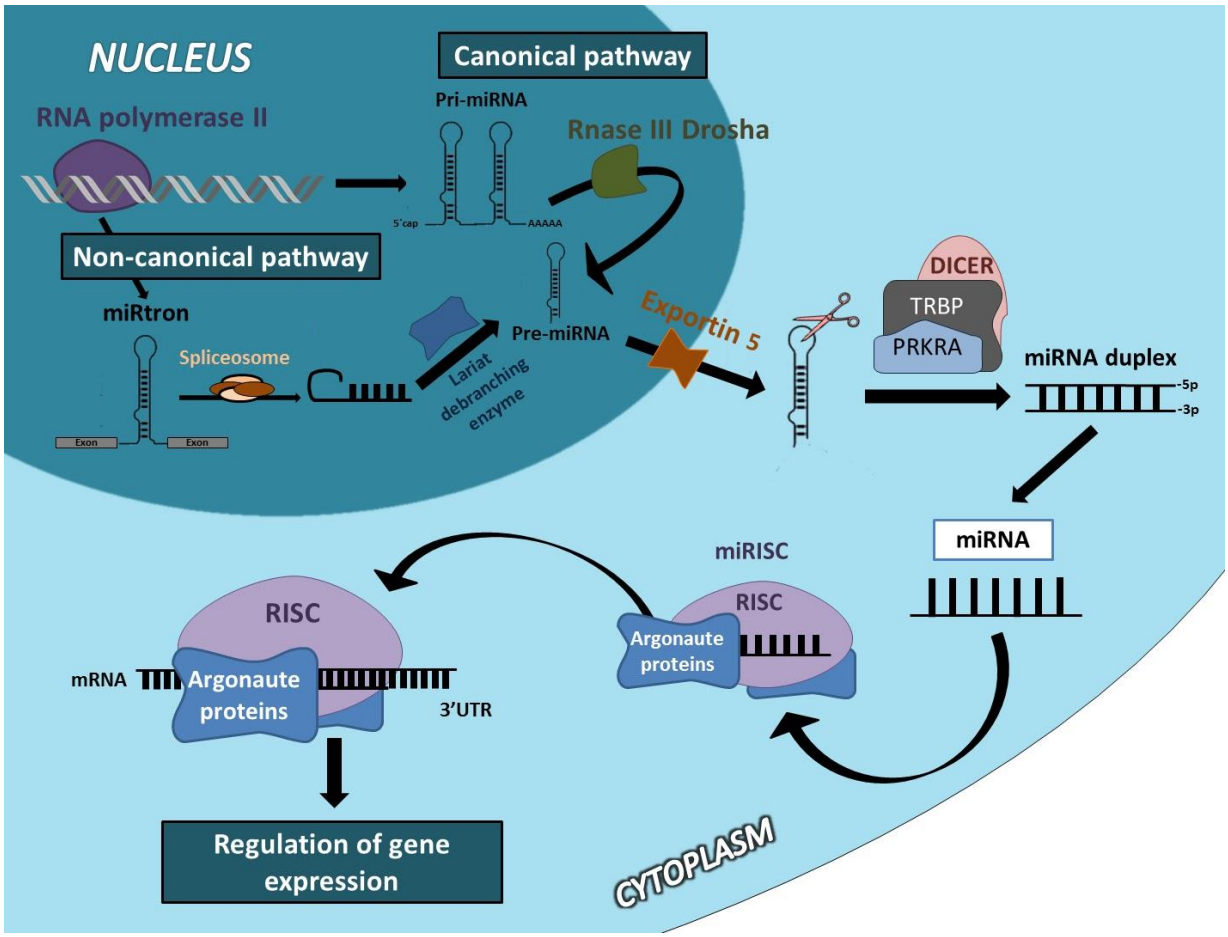


Figure 9. miRNA canonical and non-canonical biogenesis. *(Figure published in Thrombosis Research in 2018)*[191]

3.3. Mechanisms of action

It is not easy to assess the functionality of a miRNA in a whole system, mostly because one single miRNA can target several mRNA and one mRNA can be regulated by several miRNAs. The miRISC specificity for its target is due to the complementarity on the target mRNA sequence, called miRNA response elements (MRE). The degree of complementarity in this region determines if the final step is the slicing of mRNA by AGO2 or the inhibition of translation and mRNA decay mediated by miRISC[190]. In animal cells, miRNA:MRE interactions are normally not fully complementary[197] and the interaction occurs in the 5' seed region, a key sequence of the miRNA composed of 2-8 nucleotides (**Figure 10**). Most miRNAs are known to inhibit gene expression, but there is also evidence of protein up-regulation by miRNAs. The activation of translation via miRNAs involves different proteins than the inhibition, such as the Fragile-X-mental retardation related protein 1 (FXR1)[198].

One important peculiarity of miRNAs is their dynamism, which helps to maintain gene expression in a steady state. This is partially due to the subcellular compartmentalization of miRNAs. Target mRNA and miRISC localize in multiple subcellular compartments such as lysosomes, mitochondria, nucleus, rough endoplasmic reticulum, etc[199,200]. This favors miRNA to react quickly to any change in subcellular environments and, therefore, regulate its targets dynamically. Many other aspects contribute to miRNAs activity; miRNA and mRNA abundance, cell state and type, the availability of miRISC components or the affinity for MRE[192].

It is also known that circulating or extracellular miRNAs play an important part in intercellular communication. They can be secreted and shipped to recipient cells and regulate the activity of those host cells. Some can even interact with cell surface receptors, therefore acting similarly to hormones. Anyways, more *in vivo* studies must be performed to determine miRNAs specific activity under physiological conditions[192].

miRNAs as biomarkers

As described before, miRNAs bear plentiful advantages that make them ideal biomarkers in most diseases. They can be measured in blood, urine and other fluids, therefore they are easily accessible. They are also very sturdy against external threats, such as freezing and thawing, enzymatic degradation or intense pH conditions[184].

Secondly, miRNAs are highly conserved between different species, an important feature for research applications on model organisms.

They are tissue or cell type specific and they vary as the disease progresses, so they can serve as markers of progression and, for example, cancer stages[201] or response to therapy[202]. We must notice that, to use miRNAs as biomarkers in clinical practice, standardized protocols are required. Also, multiple individual characteristics (gender, age, lifestyle, etc) can affect the level of circulating miRNAs and, therefore, large sample sizes are needed for those studies. For now, numerous independent validation studies are needed to come up to clinical application[203].

Figure 10. Interaction of a miRNA and its target mRNA mediated by Ago2. Inside the green call out it is marked the ~7 nucleotides seed region. In the yellow call out it is marked the 5 or less nucleotides supplementary chamber. nt: nucleotides.

HYPOTHESES AND AIMS

HYPOTHESIS

Acute coronary syndrome is a complex disease, in whose development are involved epidemiological factors and acquired habits, with a plausible participation of genetic alterations, although the latter have been less studied. Traditionally, ACS has been considered a disease associated with age, with a development that parallels aging of the individual. This circumstance implies a pathophysiological basis for atherosclerosis, a sustained inflammatory process, atherosclerosis, of crucial importance in ACS. Younger patients, however, are considered a differentiated clinical subgroup in which plaque erosion, rather than atherosclerosis, may play a relevant role. Their distinctive features can be associated with a different clinical presentation, prognosis, evolution, and treatment.

In parallel to technological development, in recent years the participation of many different cell types in atherosclerotic plaques has been identified, incorporating the notion that innate immunity also plays a role in their progress. In this context, the most innovative vision of cardiovascular disease contemplates the interaction of three processes, inflammation, innate immunity, and haemostasis, a process defined as immunothrombosis. These new concepts have been established in several series of older patients, but it is not known whether they are equally involved in younger patients with ACS.

Our hypothesis is that ACS in young patients is also a disease with an important thromboinflammatory component. In addition, once the confounding factors inherent to aging are controlled, the involvement of genetic factors will be more evident. In the search for genetic alterations, we explored variants in miRNA genes, which, being regulators of inflammatory and atherothrombotic processes, may be associated with the onset, severity, and/or evolution of ACS in younger patients.

MAIN AIM

The central objective of this research is to determine whether ACS in young patients is a thromboinflammatory disease and to identify genetic variants in miRNA genes that can be considered as biomarkers of thromboinflammation and/or of the occurrence and recurrence of ACS in these patients.

Aim 1. To study the association of miR-146a rs2431697 genotype with the clinical outcomes of ACS young patients through exacerbated NET production.

Aim 2. To investigate if the overexpression of TF in response to NET components, histones, and DNA, is mediated by miRNA regulation.

Aim 3. To identify new variants in miRNA genes with a potential pathological role in the onset of ACS.

CHAPTER 1

ROLE OF miR-SNP rs2431697 IN YOUNG PATIENTS (≤ 45 YEARS) WITH ACUTE CORONARY SYNDROME

The research presented below was published in the Journal of Personalized Medicine in July of 2022 [204]

1. INTRODUCTION AND BACKGROUND

ACS and thromboinflammation in young patients

The incidence of ACS has decreased in the last decades thanks to the improvements in diagnosis, therapeutics strategies and, even, changes of lifestyle in the older population. But in younger patients, this decrease is not that significant. Actually, the prevalence of comorbidities has risen over the past decades in these patients[205]. One likely explanation is that ACS at young ages is robustly influenced by genetic factors, therefore, less modifiable with lifestyle changes[47].

The etiopathogenesis of ACS has suffered notorious conceptual changes during the last decades due to research in older patients. We know that thrombus composition is deeply heterogeneous, varying among patients and thrombotic disorders. This heterogeneity will determine the responses to treatments and the outcomes of the disease[206].

Lastly, inflammation has become a new important element in the context of ACS and even as a therapeutic target[207]. The relationship between innate immunity and blood coagulation may have prothrombotic consequences in a process known as immunothrombosis. Its dysregulation, known as thromboinflammation, is a major element in the development of thrombotic events that take place in sterile inflammatory diseases such as stroke, AMI, and disseminated intravascular coagulation. This concept is interesting in the field of therapeutics strategies, since some of the immunothrombosis mediators are less indispensable in haemostasis, therefore, targeting thromboinflammation open new options of treatment and prevention of thrombotic disorders[119]. Mechanistically, neutrophils migrate to the nascent thrombus, after their interaction with platelets, where they contribute to the propagation of the thrombus via the formation of NETs and their proinflammatory and cytotoxic effects[154]. These new elements add so much complexity to cardiovascular diseases that the classical model with only one type of atherosclerosis is becoming obsolete[208].

It is known that, in acute cardiovascular disease, NETs are present in the ischemic myocardium and the NETs burden in STEMI patients' coronary thrombi correlates with the infarct size[152]. Moreover, NET components have been studied as a marker of ACS or of major adverse cardiac events (MACE)[209–211]. Even so, before our present study, there was no data about plasma NET components in young ACS.

Role of miR-146a as thromboinflammatory mediator

In this context, our group has described the role of another element involved in immunothrombosis through NETs; miR-146a[104]. This miRNA was described for the first time in 2006, in an acute monocytic leukemia cell line, and is known to have a role in atherosclerosis since it is capable to negatively regulate pro-inflammatory factors such as TNF receptor-associated protein factor 6 (TRAF6), IL-1 receptor-associated kinase 1 (IRAK1), and TLR4[212,213]. Furthermore, in SLE patients, decreased levels of miR-146a in neutrophils are linked with the thickening of carotid intima-media[214] (**Figure 11**).

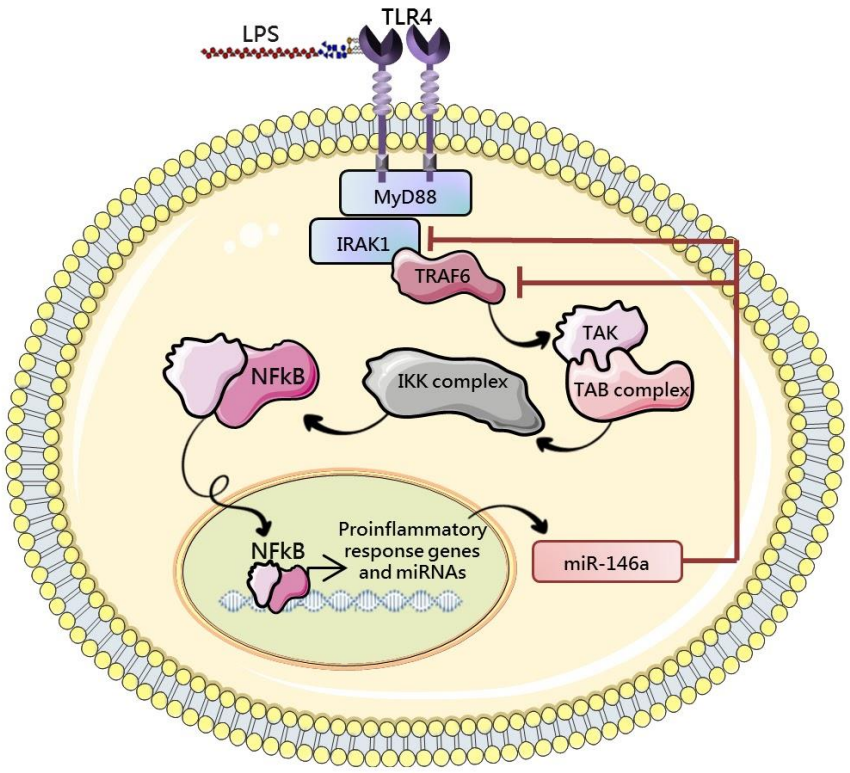


Figure 11. Regulation of the TLR4 / NF-κB axis via miR-146a. *(Figure published in Thrombosis and Haemostasis in 2021)* [218]

In several previous reports we have demonstrated the functional role of miR-146a in thromboinflammation. Firstly, we showed that the deficiency of miR-146a in hematopoietic lineage did not affect atherogenesis after 8-weeks of high fat diet in a low-density lipoprotein receptor knock-out (*Ldlr*$^{-/-}$) mice model[215]. In addition, we demonstrated that NE activity can provide new information for MACE prognostic in atrial fibrillation patients, with a potential role of miR-146a genotype in NET generation

and cardiovascular risk[216] (**Figure 12**). We further explored the *in vivo* consequences of such associations in an arterial thrombosis model via $FeCl_3$ injury in miR-146a$^{-/-}$ mice *vs*. wild type (WT) mice. We found increased levels of NETosis in the thrombi of deficient mice as well as a shorter time for vessel occlusion. These findings were similar to those from a non-sterile inflammation model via LPS injection in these deficient mice[217] (**Figure 12**). All these results are sustained by the fact that miR-146a plays a relevant role in macrophages polarization, since its deficiency promotes the phenotype M1 due to the increase of NOTCH1 expression. Also the direct repression of TLR4 (main receptor of PAMPs in myeloid cells), by this miRNA has been widely characterized in several pathologies[218–221] (**Figure 13**).

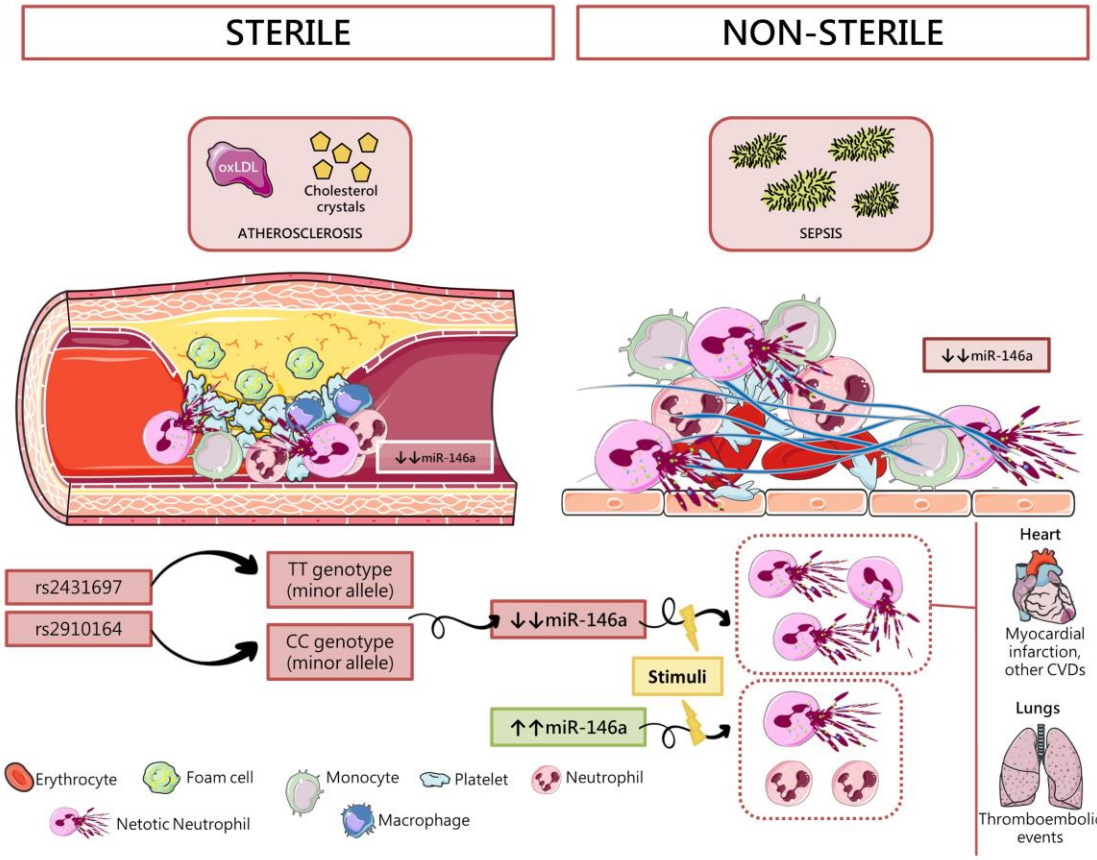


Figure 12. Schematic representation of the role of miR-146a in thromboinflammation. The SNPs rs2431697 and rs2910164 are associated (the minor allele) with a phenotype with lower levels of miR-146a. Those neutrophils, upon sterile or non-sterile stimuli will cause more NETosis and, therefore, a worse outcome of thromboinflammatory diseases. *(Figure published in Thrombosis and Haemostasis in 2021)* [218]

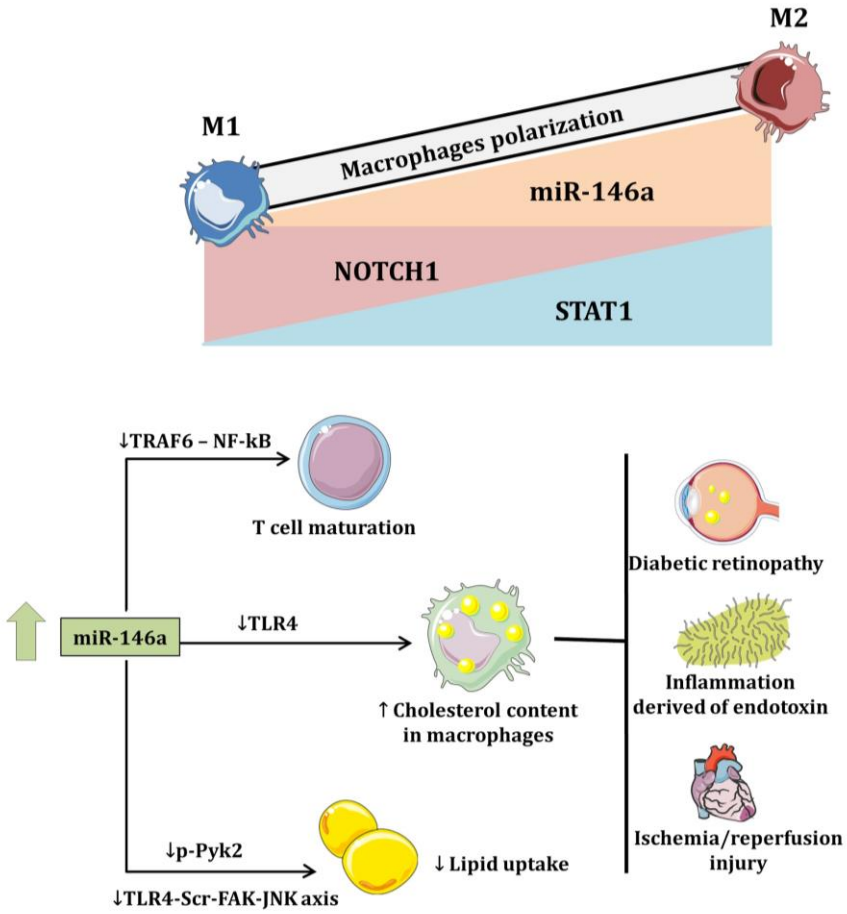


Figure 13. miR-146a deficiency promotes macrophage polarization to M1 phenotype. Conversely, miR-146a overexpression promotes M2 phenotype by decreasing the expression of NOTCH1 and inhibits the differentiation to M1 by targeting STAT1. miR-146a overexpression can block the TLR4-Src-FAK-JNK axis and inhibit the Pyk2 phosphorylation leading to the inhibition of lipid uptake. This overexpression can also decrease the NF-κB activity through repressing TRAF6, resulting in T cells maturation. By provoking a lower TLR4 expression and higher intake of cholesterol by macrophages, the high levels of miR-146a may finally lead to diabetic retinopathy, inflammation derived from endotoxin or ischemia/reperfusion injury, among other pathologies. *(Figure published in Thrombosis and Haemostasis in 2021)*[218]

One feature that makes miR-146a especially interesting is the presence of single nucleotide polymorphisms (SNPs) able to regulate its expression. Genome-wide association studies (GWAS) performed in the past decade identified several miR-SNPs associated with this miRNA, such as: rs2910164, associated with papillary thyroid cancer susceptibility[222]; rs57095329, located in the promoter region of the primary transcript of *miR-146a* and associated with SLE in a Chinese population[223]; rs2277920,

in linkage disequilibrium with the previous SNP, but not related with the susceptibility to SLE and rs2431697, in which we will focus in this study[224]. (**Figure 14**)

rs2431697 is a SNP upstream of miR-146a, it was identified in 2008 in a GWAS in European population as a susceptibility marker for SLE[225]. It is located in an intergenic region between *PTTG1* and *MIR146A* with a minor allele frequency (MAF) of 0.27. The T allele of this SNP, considered as the risk allele, caused a decrease of 1.6-fold in the expression levels of miR-146a, while it showed no effect in *PTTG1 expression*[224]. Our group has reported that the rs2431697 TT genotype is an early predictor of myelofibrosis progression independently of the JAK2V617F allele burden[226] and, also, that rs2431697 genotype is a marker of MACE in atrial fibrillation patients[227,228].

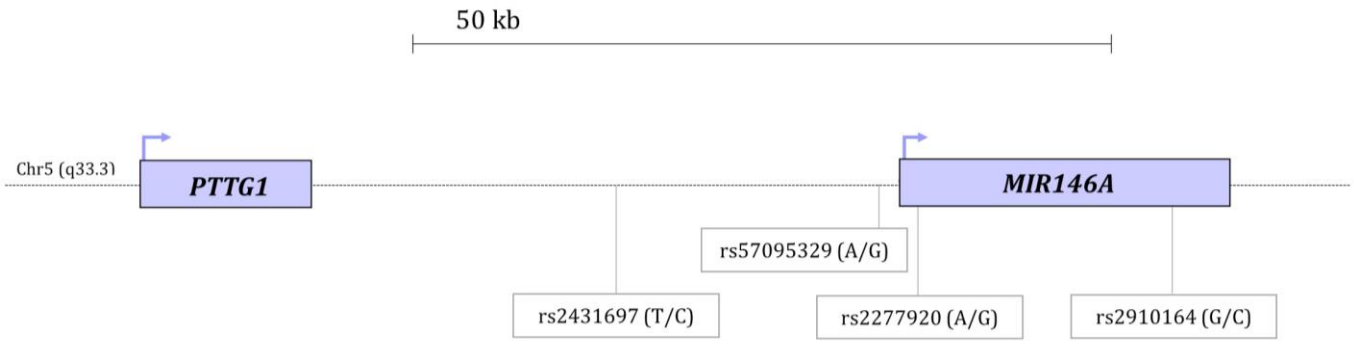


Figure 14. Single nucleotide polymorphisms identified in the*MIR146A* gene and near it.

We found in this polymorphism the perfect model of partial and physiological deficiency of miR-146a. We showed that the activation *in vitro* of neutrophils from TT genotype individuals for rs2431697 produced a higher NET release than C-allele carriers and the combination of NETs markers and rs2431697 genotype are useful as new prognostic markers of adverse events in atrial fibrillation[216,229], AF, and community-acquired pneumonía patients[217,218] (**Figure 12**).

With all this background information, we considered pertinent to study the role of miR-146a in young patients with ACS. We investigated the association between NETosis markers and clinical outcomes of ACS patients and their link with miR-146a rs2431697 genotype, as well as the prognostic value of those elements.

2. STUDY POPULATION

2.1. Patients and donors

Patients with ACS history under 45 years of age were enrolled between January 2015 and March 2020. The inclusion criterion for patients with recurrent ACS was that the first event had happened before the age of 45. We did not establish any other specific exclusion criteria in order to reflect the "real-life" clinical practice.

Due to the heterogeneity of the population and in order to perform specific analysis, patients were classified in three groups. Group 1 (G1) composed of patients that suffered ACS being younger than 45 years of age and the sample was collected in the context of that hospitalization. Group 2 (G2), patients that suffered a first ACS being younger than 45 years, but the samples were collected within 90 days of suffering a recurrent ACS event afterwards. Finally, group 3 (G3) includes patients that suffered ACS before being 45 years old and have no recurrence; they were recruited retrospectively (**Figure 15**).

To complete the study, we recruited 300 healthy donors, matched in age and sex with the patients, as our control group. Written informed consents were provided for all individuals and the study was authorized by the Ethics Committee of Hospital Clínico Universitario Virgen de la Arrixaca and Hospital General Universitario Morales Meseguer in Murcia, Spain.

2.2. Follow-Up and Endpoints

All patients were followed-up for 2 years after collection, all adverse events were recorded during this period. All clinical outcomes were identified, confirmed and recorded and the primary endpoints were ischemic events (STEMI, NSTEMI, and stent thrombosis), cardiovascular hospitalization, any cardiovascular outcome, and all-cause mortality.

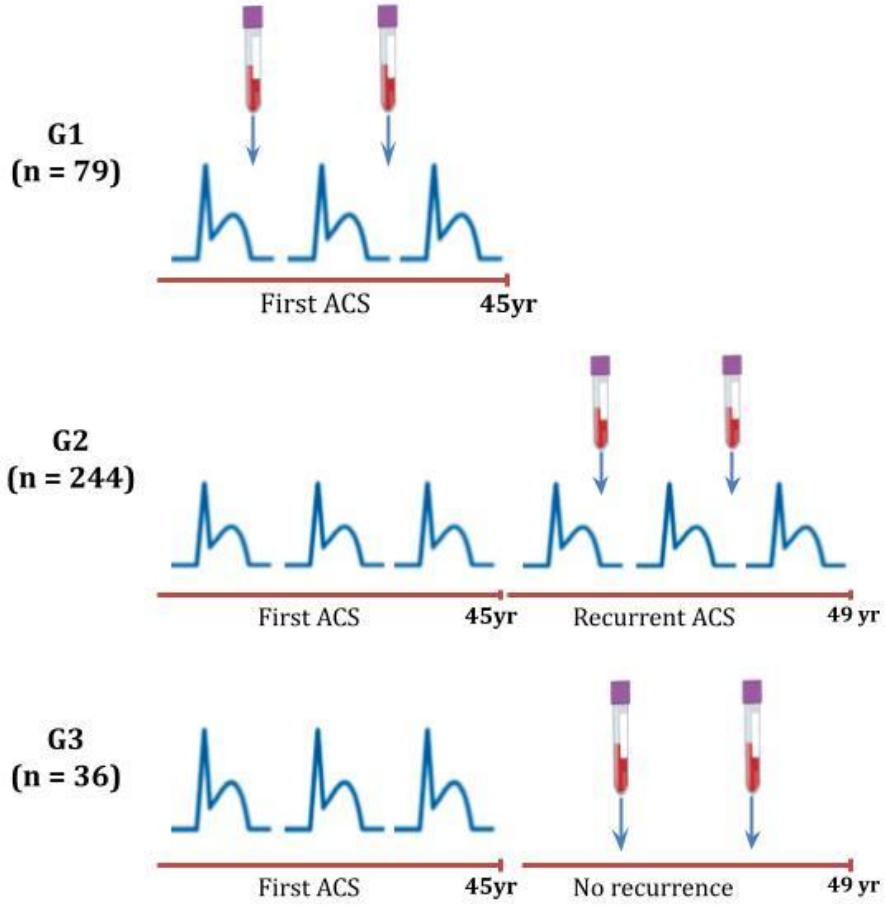


Figure 15. Graphical description of the classification of patients in groups according to the recurrence of time from the event.

3. MATERIALS AND METHODS

3.1. Genomic DNA extraction

Blood was drawn in EDTA tubes for all patients and donors. These samples were centrifuged at 2500x g during 15 minutes at room temperature (RT) within 24 h after collection. Once centrifuged, the buffy coat was used for DNA extraction using the QIAcube device and QIAamp DNA Blood Mini Kit (Quiagen, Madrid, Spain) and following the manufacturer instructions. Quantification and quality testing (A260/A280 and A260/A230 ratios) of total DNA were performed with NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). Finally, DNA samples were stored until their analysis at -20ºC.

3.2. cfDNA quantification

Double stranded cfDNA was quantified in plasma samples using Sytox Green (ThermoFisher Scientific), a fluorescent nucleic acid stain, following the already described protocols[230]. The cfDNA concentration was calculated from a standard curve made with known concentrations of salmon sperm DNA (Sigma-Aldrich, Madrid, Spain). In 96-well dark plates, the plasma was diluted 1:3 in Tris buffered saline (TBS) and an equal volume of 2 μM Sytox Green was added and incubated for 15 minutes at RT in the dark. The fluorescence was analyzed at 488 nm (excitation) and 528 nm (emission) in a plate reader (Biotek Synergy Ht, Izasa, Barcelona, Spain). Every sample was also incubated without Sytox Green as autofluorescence control.

3.3. CitH3-DNA complexes quantification

For quantification of CitH3 and DNA complexes colocalized (citH3-DNA), we followed the technique proposed by Lefrançais et al[231], consistent in a sandwich ELISA. Rabbit anti-citH3 (citrulline R2 + R8 + R17, ab-5103, Abcam, Madrid, Spain) was the capture antibody and was coated on the plate overnight. After that, the wells were washed and blocked. Citrated plasma samples, or phosphate-buffered saline (PBS) as a negative control, were added to the plate and incubated for 2h at RT. Then, wells were washed again and incubated during 2h at RT with the marked secondary antibody; peroxide-conjugated anti-DNA antibody from the Cell Death Detection ELISA Kit (Roche Applied Science, Indianapolis, IN, USA). Finally, we added the peroxidase substrate ABTS (ThermoFisher Scientific, Madrid, Spain) and measured the optical density (OD) at 450 nm for 2h in a spectrophotometer. The results were represented as 450 nm absorbance, since there is no available standardized control of known concentration (**Figure 16**).

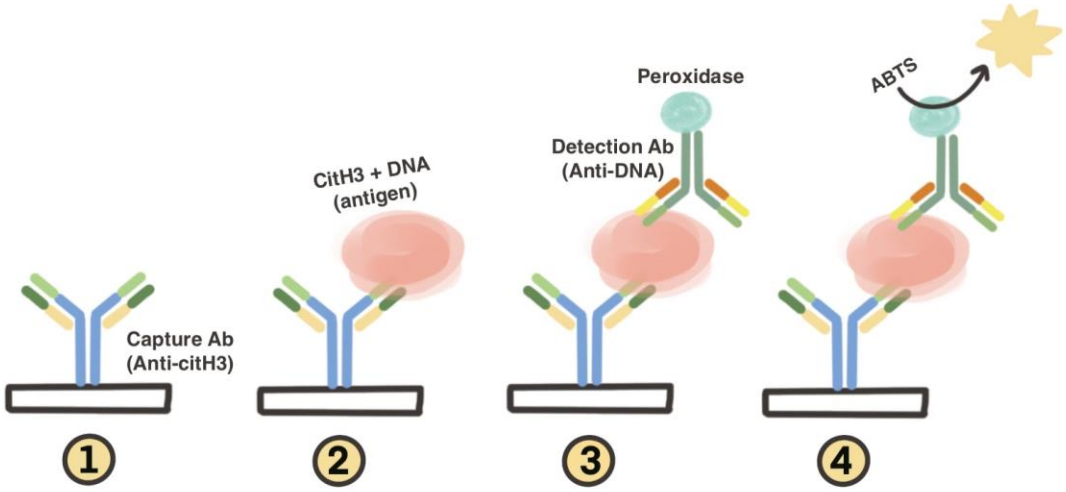


Figure 16. Overview of the sandwich ELISA technique. **1-** The capture antibody was getting attached to the plate well and ready to recognize the citH3. **2-** Plasma supposedly containing the NETs components, citH3-DNA, was added to the plate and citH3 was recognized by the capture antibody. **3-** The detection antibody was added and recognized the DNA that was present in plasma and attached to the anti-citH3 antibody. **4-** The peroxidase substrate ABTS was added causing the coloration that was measured by the spectrophotometer. Ab: Antibody.

3.4. miR-146a genotyping

The miR-SNP rs2431697 of miR-146a was genotyped using an available commercial Taqman probe; *C_26633319_10* (ThermoFisher Scientific, Madrid, Spain), we optimized the reaction for the use of 0.2 µL of probe, 2.5 µL of Premix ExTaq 2x buffer (ThermoFisher Scientific, Madrid, Spain), 0.3 µL of DNase-free water, and 1 µL of DNA (20 ng/µL) leading to a final volume for the reaction of 5 µL. The DNase-free water was also used as a negative control without DNA. The assay was performed in a LightCycler 480 (Roche Farma, Madrid, Spain).

3.5. Statistical analysis

Categorical variables are expressed as absolute frequencies and percentage, while continuous variables are expressed as mean ± standard deviation (SD) or median and interquartile range (IQR) as appropriate.

Pearson chi-squared test was used when comparison of proportions was needed. Mann-Whitney U test or Student t-test were used for assessed differences between continuous and categorical variables, as appropriate. Spearman's Rho or Pearson's correlation coefficient was used for assessing correlations. To determine the association between rs2431697 genotype, NET markers and the risk of recurrent events we used logistic regression models. Results are presented as odds ratios (ORs) with 95% confidence intervals (CIs). We considered a *p*-value < 0.05 as statistically significant. Statistical analysis were performed using SPSS version 25.0 (SPSS, Inc., Chicago, IL, USA).

4. RESULTS

4.1. Clinical features of the study population

A cohort of 359 ACS patients was enrolled. They were mostly males (88%) with a median age of 44 years old (IQR 40-47). The main characteristics of these patients are presented in **Table 2**. From the overall cohort, it is worth noting that 13 (3.6%) had unstable angina, 212 (59.1%) presented STEMI and 134 (37.3%) had NSTEMI. We found differences in risk factors between healthy donors and ACS patients, but there were no differences in sex or age.

The clinical characteristics of each group of patients are presented in **Table 3**. We did not find relevant differences either in demographic data or in rs2431697 frequencies between the different groups.

4.2. NETosis markers in ACS patients and healthy controls

Both, citH3-DNA complexes and cfDNA, measured in plasma, were robustly increased in ACS patients when compared to healthy donors (0.296 *vs*. 0.215 O.D. (n = 343 / n = 51) and 0.668 *vs*. 0.414 ng/µL (n = 342 / n = 55); both markers with $p < 0.0001$) (**Figure 17 - A, B**). We also found correlation between cfDNA and citH3-DNA complexes (r = 0.389; $p < 0.0001$).

Focusing on groups of patients, there were no differences in cfDNA levels between them (**Figure 17 - C**). Per contra, citH3-DNA levels were significantly higher in G1 compared to G2 (0.227 *vs*. 0.148 ng/µL (n = 69 / n = 239), $p< 0.01$), while G3 showed no differences with those groups (**Figure 16 - D**).

Table 2. Age and sex, main clinical characteristics and rs2431697 genotype of the ACS cohort *vs* healthy donors.

	Healthy donors (n = 300)	**ACS Patients (n = 359)**	***p*-Value**
Age, mean (range)	42 (22-45)	44 (22-46)	0.152
Male sex, N (%)	251 (83.7)	316 (88.0)	0.115
STEMI, N (%)	-	212 (59.1)	-
NSTEMI, N (%)	-	134 (37.3)	-
Unstable angina, N (%)	-	13 (3.6)	-
Obesity, N (%)	25 (8.3)	124 (34.5)	<0.0001
Hypertension, N (%)	26 (8.7)	91 (25.3)	<0.0001
Diabetes, N (%)	6 (2.0)	48 (13.4)	<0.0001
Dyslipidemia, N (%)	61 (20.3)	121 (33.7)	<0.0001
Current smoking, N (%)	41 (13.7)	255 (71.0)	<0.0001
rs2431697 genotype			
CC, N (%)	53 (17.7)	60 (18.7)	0.689
CT, N (%)	141 (47.0)	154 (42.9)	0.329
TT, N (%)	71 (23.7)	105 (29.2)	0.106
C Allele, N (%)	194 (64.7)	214 (59.6)	0.183
T Allele, N (%)	212 (70.7)	261 (81.3)	0.563

NSTEACS: non-ST segment elevation acute coronary syndromes. NSTEMI: non-ST segment elevation myocardial infarction. STEMI: ST segment elevation myocardial infarction. Genotype was available in 265 controls and in 321 ACS

Table 3. Age, sex, main clinical characteristics and rs2431697 genotype of the ACS cohort within each group at the time of the index event.

	G1 (n = 79)	**G2 (n = 244)**	**G3 (n = 36)**	***p*-value**
Age, mean ± SD	42(39-44)	45 (42-49)	41.5 (39-43)	0.305
Male sex, N (%)	63 (79.7)	218 (89.3)	35 (97.2)	0.052
STEMI, N (%)	44 (55.7)	140 (57.5)	28 (77.8)	0.053
Obesity, N (%)	21 (26.6)	79 (32.4)	13 (36.1)	0.603
Hypertension, N (%)	21 (26.6)	64 (26.2)	6 (16.7)	0.412
Diabetes, N (%)	10 (12.6)	33 (13.5)	5 (13.9)	0.922
Dyslipidemia, N (%)	25 (31.6)	82 (33.6)	14 (38.9)	0.814
Current smoking, N (%)	50 (63.3)	177 (72.5)	28 (77.8)	0.316
rs2431697 genotype				
CC, N (%)	12 (15.2)	43 (19.8)	5 (15.6)	0.745
CT, N (%)	34 (43)	102 (42.1)	19 (52.8)	0.749
TT, N (%)	26 (32.9)	72 (29.8)	8 (22.2)	0.683
C-allele, N (%)	46 (58.2)	145 (59.9)	24 (66.7)	0.907
T-allele, N (%)	60 (83.3)	174 (80.2)	27 (84.4)	0.642

NSTEACS: non-ST segment elevation acute coronary syndromes. STEMI: ST segment elevation myocardial infarction. Genotype was available in G1= 72, G2= 217 and G3= 32

4.3. Relationship between rs2431697 and ACS

As expected, the distribution of rs2431697 genotypes between ACS patients and healthy individuals was not statistically different (**Table 2**), therefore the presence of the SNP does not determine the onset of disease. The distribution was in accordance with Hardy-Weinberg equilibrium (ACS: p = 0.695; healthy controls: p = 0.424). There were no differences between the different groups of patients (**Table 3**).

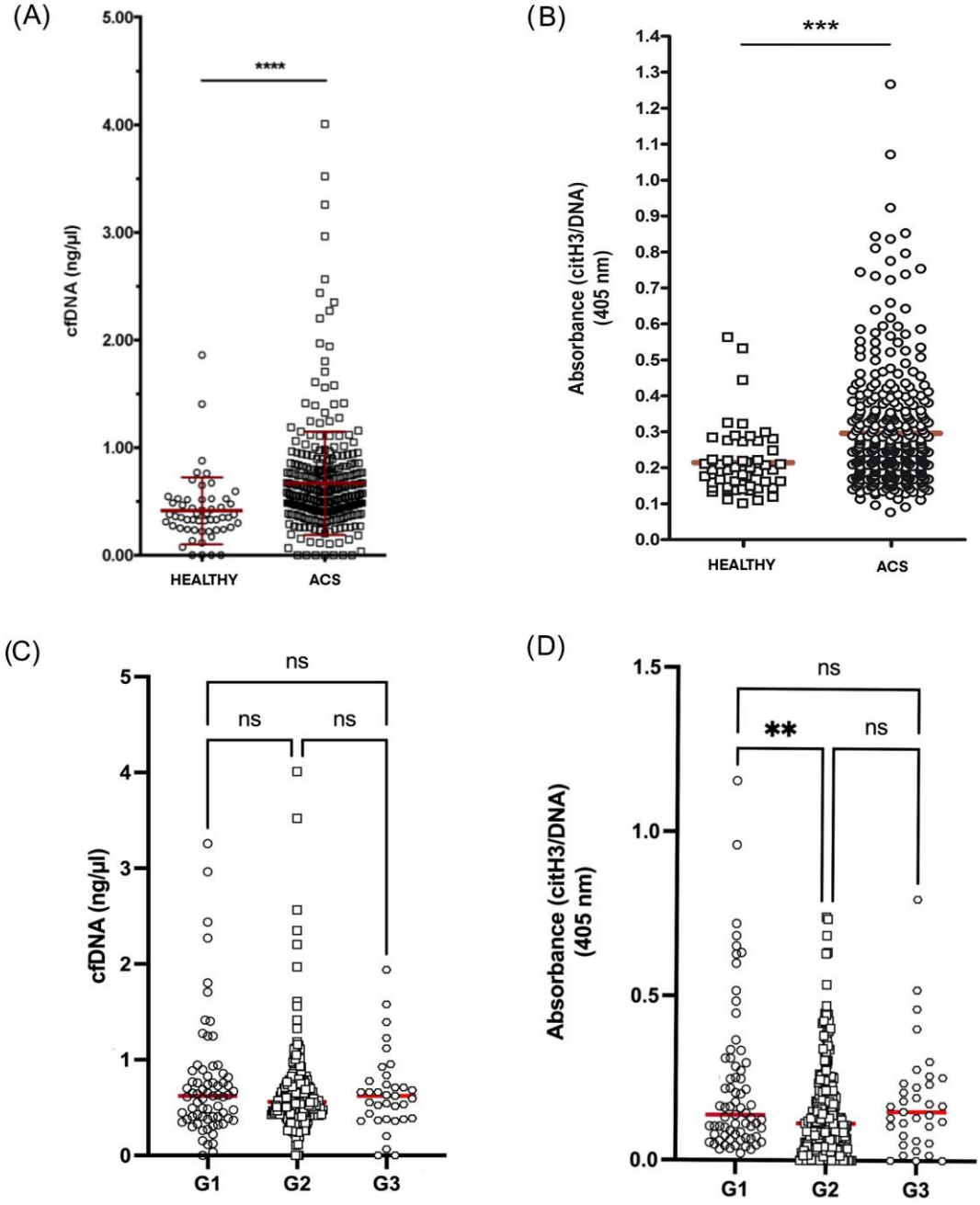


Figure 17. Levels of NETs markers in ACS patients younger than 45 years old. **A -** cfDNA levels (ng/µL) in healthy controls (n = 55) *vs*. ACS patients (n = 342) measured by fluorescence of Sytox Green reagent. **B -** citH3-DNA levels presented as absorbance levels in healthy donors (n = 51) *vs*. ACS patients (n = 343) quantified by ELISA. **C -** cfDNA levels (ng/µL) in young ACS patients classified by group (G1 n = 69; G2 n = 238; G3 n =35). **D -** citH3-DNA complexes, presented as absorbance at 405 nm, between the different groups of patients (G1 n = 69; G2 n = 239; G3 n =35). ns: not significant; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

4.4. rs2431697 and NETosis markers in ACS patients

We studied the association between allelic frequencies and cfDNA or citH3-DNA complexes in the complete cohort of patients comparing all possible genotypes and found no differences between them (**Figure 18**). However, when we grouped the genotypes by the risk allele, and focusing on ACS patients, we found that T allele carriers were higher than C-homozygous patients (cfDNA: 0.654 *vs*. 0.540 ng/µL; p = 0.015. citH3-DNA: 0.304 *vs*. 0.265 OD; p = 0.041. CC n = 56; CT+TT n = 231). In accordance with our previous studies, no association was found between the rs2431697 genotype and any of the two markers in healthy individuals (**Figure 19 - A, B**).Analyzing the carriers of the risk allele by group of patients, we found again significant differences in citH3-DNA levels between G1 and G2 (0.224 *vs.* 0.154 OD; $p < 0.05$; n = 58 / n = 196), but none between other groups or in cfDNA levels (**Figure 19 - C, D**).

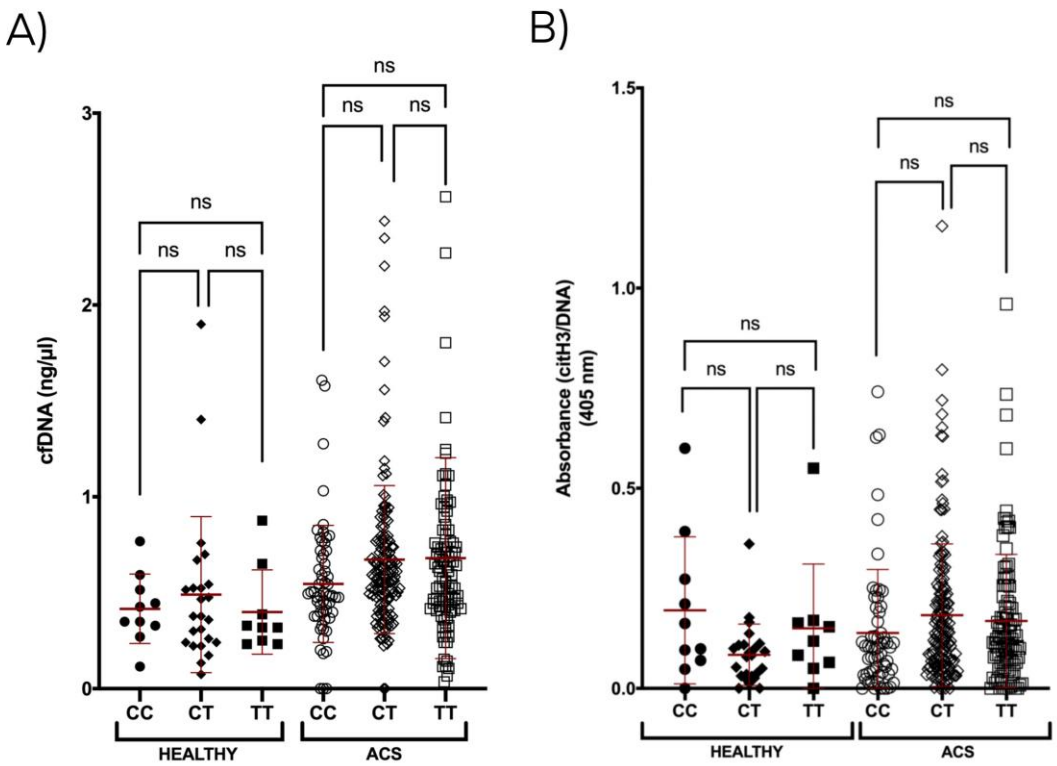


Figure 18. Levels of NETs markers in ACS patients younger than 45 years old between different alleles of rs2431697. **A -** cfDNA levels (ng/µL) in healthy individuals (n = 42) and ACS patients (n = 342). **B -** citH3-DNA complexes (absorbance at 405 nm) in healthy donors (n = 42) and ACS patients (n = 343).

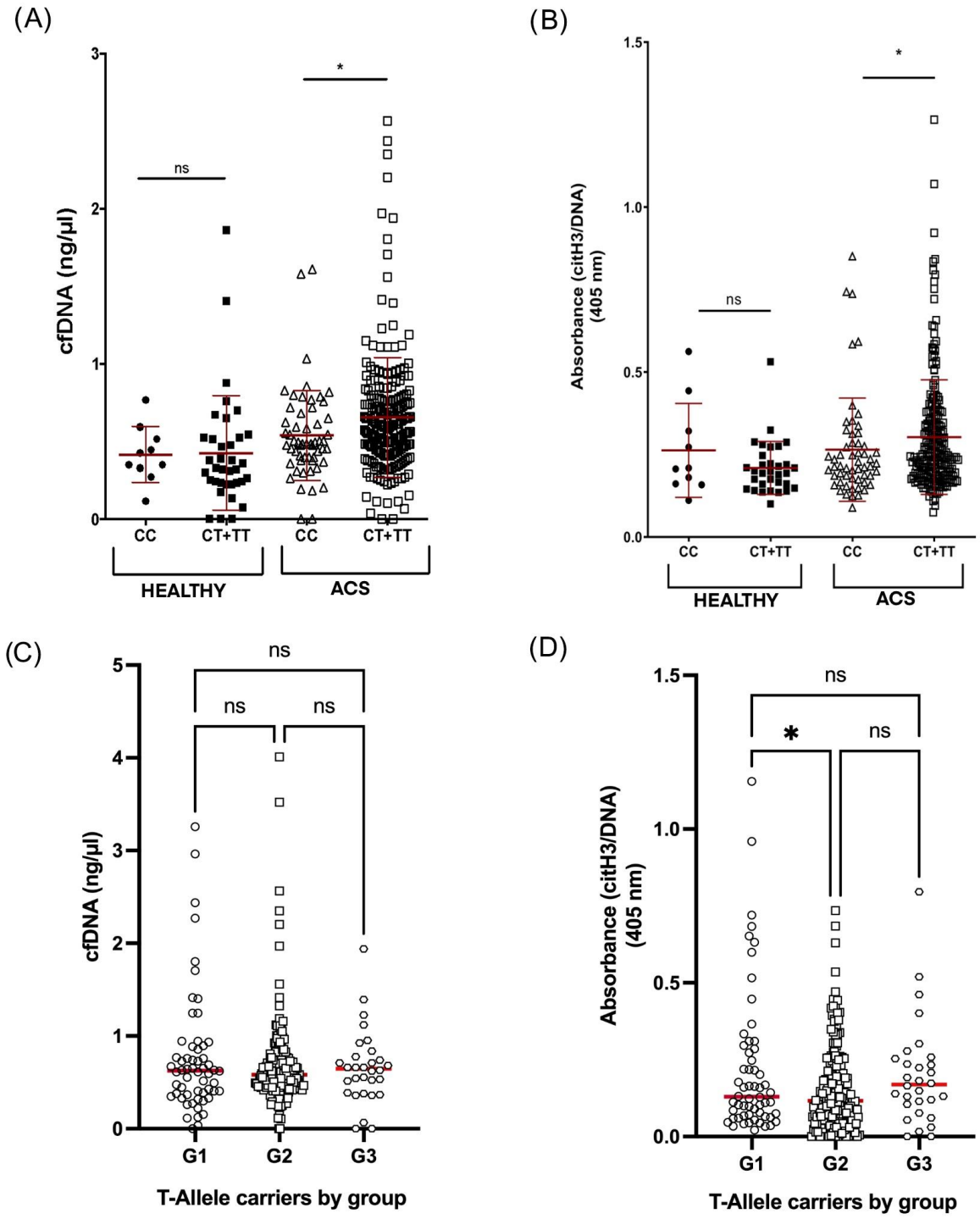


Figure 19. Levels of NETs markers in ACS young patients by rs2431697 genotype. **A, B -** cfDNA levels (ng/µL) and citH3-DNA complexes levels (absorbance at 405 nm) in healthy controls and ACS patients carrying or not the risk allele of rs2431697 (CC n = 56; CT+TT n = 231). **C, D -** cfDNA levels (ng/µL) and citH3-DNA complexes levels (absorbance at 405 nm) in patients carrying the T allele of rs2431697 by group of patients (G1 n = 58; G2 n = 196; G3 n = 30). ns: not significant; * $p < 0.05$.

4.5. Associations between NETosis markers and the outcome of ACS

Studying the overall cohort of patients, we found positive and significant, but moderate, correlations between cfDNA or citH3-DNA levels and the Killip-Kimball score (Pearson's r = 0.201, $p < 0.001$ and r = 0.173, $p = 0.002$, respectively).

Supporting the link between NET markers and clinical outcome, we found that patients with citH3-DNA complexes levels above Q4 (OD > 0.219) presented higher prevalence of stroke history when compared with the rest of patients (5/82 (6.1%) *vs*. 4/250 (1.6%), *p = 0.026)* (**Table 4**).

Table 4. Prognostic value of NETosis markers in the overall cohort of young ACS patients.

Pearson's correlation		
cfDNA	Killip-Kimball score	r = 0.201; $p = 0.001$
citH3-DNA complexes	Killip-Kimball score	r = 0.173; p = 0.002
Chi-square		
citH3-DNA complexes ≥ Q4	Previous stroke	$X^2 = 4.928$; $p = 0.026$

4.6. Predictive ability of NETosis markers combined with rs2431697 for adverse cardiovascular events in ACS young patients

Lastly, we analyzed if NET markers in combination with the T allele of rs2431697 genotype, were able to identify the patients with higher risk of recurrent ischemic events. As described before, patients were followed up for two years. For this analysis, we considered as high risk those patients that were carriers of the T allele and had both NETosis markers above the median (Q2) (cfDNA > 0.573 ng/μL and citH3-DNA OD > 0.116, respectively). With this analysis, we did not find a significant increase in the risk of new events for the overall cohort (OR: 1.23; 95% CI: 0.72-2.12, $p = 0.452$). However, patients in G2, the group with an actual recurrent event, presented higher risk of recurrence for the combination considered (OR: 2.09; 95% CI: 1.10-3.97, $p = 0.024$). We found no increase in the risk of new events in the rest of groups fulfilling the criteria, the data are presented in **Table 5**.

Table 5. Associations of combination of citH3-DNA and cfDNA above Q2 and rs2431697 risk allele with the risk of recurrent cardiovascular events.

Patient Group	Criteria	OR; 95% CI *	*p*-Value
Overall cohort	citH3-DNA > Q2 + cfDNA > Q2 + rs2431697 T allele	1.23; 0.72-2.12	0.452
G1	citH3-DNA > Q2 + cfDNA > Q2 + rs2431697 T allele	0.38; 0.04-3.48	0.391
G2	citH3-DNA > Q2 + cfDNA > Q2 + rs2431697 T allele	2.09; 1.10-3.97	0.024
G3	citH3-DNA > Q2 + cfDNA > Q2 + rs2431697 T allele	-	-

* Risk of recurrence at 2 years of follow-up.

5. DISCUSSION

In 2015, for the first time, a study described that the load of NETs in culprit coronary thrombi was highly related to the size of the infarcted area and to the resolution of the ST-segment[152], after that, many groups have studied the dual utility of NETs components in cardiovascular diseases, as therapeutic targets but, specially as biomarkers with the potential to define the biology of the cardiovascular outcome[145,207]. Those studies were performed in classical, old patients; as far as we know, there are no data about NET components related to the clinical outcome of ACS in younger patients.

Here, we investigated the thromboinflammatory background in young patients, evaluating NETosis markers, citH3-DNA complexes and cfDNA, and rs2431697 genotype in relation to the onset and recurrence of ACS.

Regarding NET markers in plasma from young ACS, we demonstrated that cfDNA and citH3-DNA complexes were significantly higher in young ACS patients when compared to healthy donors, indicating that NETosis is increased in young ACS subjects. Since all samples were taken after ACS, we were not able to establish if NETs were the cause or the consequence of the event. NET markers' utility is controversial in cardiovascular disease. There are several factors that may influence the interpretation of their presence, such as the time elapsed since the event or where the NETs are quantified. While there is a consensus that NET markers are elevated at the site of lesion, there are contradictions in peripheral locations. For instance, it has been described that these markers are higher in coronary circulation than in plasma in STEMI

patients[210,232]. Also, Stakos et al. compared intracoronary samples from pathological and normal angiographies and found higher NETosis markers levels only in the pathological ones[121].

The specificity of the markers used for measuring NETosis (normally, cfDNA, citH3, MPO or complexes such as MPO-DNA and citH3-DNA) can also add a level of confusion. The most specific being the complexes, but they may lose usefulness at a distance of the injured zone[210]. Langseth et al. suggested that these markers in different locations could indicate distinct degrees of inflammation amid the periphery and the thrombus[233]. We found that both, cfDNA and citH3-DNA complexes, could work as useful markers of NETosis in the plasma of these patients. Therefore, we hypothesize that ACS before the age of 45 years could be the consequence of a premature inflammatory environment that predisposes to NETosis. Whereas, in older patients, aging and the inherent medication could contribute to the obscureness in the role of plasma NETs markers.

It is worth noting that there is some controversy regarding the validity of NET levels beyond the acute event. It has been stated that cfDNA behaves as a stable marker over time[234] and we found that cfDNA levels were similar in the different groups of ACS patients, in agreement with that statement. On the contrary, our data suggest that citH3-DNA, although remaining higher than in healthy subjects, could decrease over time in the patients. They were significantly elevated in G1 when compared to the rest of patients, indicating higher levels in the samples taken during the onset of ACS. These results are in line with the TATICS-TIMI18 trial, where MPO levels were linked with a greater risk of recurrence at 30 days, but this link was lower at 6 months[235].

To analyze the association between ACS, NETs and rs2431697 genotype, after studying NETs components in ACS, we investigated if miR-146a rs2431697 genotype was involved in ACS before the age of 45 years. In Chinese patients, rs2431697 genotype was described to be a risk factor for coronary artery disease[236], but our data revealed that this SNP is not related to the onset of ACS in our cohort. Demographic and acquired risk factors as well as the differences in genetic background might explain this discrepancy.

Our data showed that ACS patients carriers of the T allele of rs2431697 had significantly higher levels of cfDNA and citH3-DNA complexes than CC patients.

Therefore, demonstrating for the first time that rs2431697 is related to NETs generation in plasma of young ACS patients. We had already described this association in the worst outcome of patients with atrial fibrillation[216] and in cardiovascular events of patients with community-acquired pneumonia[217]. With the present data, we suggest that, in fact, rs2431697 may act as a genetic thromboinflammatory marker of adverse cardiovascular events in any inflammatory disease. Nevertheless, as shown in healthy individuals that do not suffer an increase in NETosis levels in their plasma, this genetic marker needs to be "primed". Then, the inflammatory stimuli, such as classical risk factors, will trigger the dysregulation revealing the functional effect of rs2431697. Within the same rationale, we have previously described that monocytes from healthy individuals, after LPS activation, transcribed more *IL6* mRNA in TT-carrier individuals than in CC-carrier ones[227]. To further support our results, the hypothesis that miRNA has a functional effect when a pathological context appears has already been postulated[237].

To complete this research, we studied the relationship between thromboinflammatory markers and clinical outcomes. We found a significant but moderate association amid elevated NETs plasma markers and severity of the disease. Both, cfDNA and citH3-DNA levels, were significantly associated with the Killip-Kimball score; it allows the establishment of a prognosis of the evolution and probabilities of death in the first 30 days after the event. Likewise, high cfDNA levels in plasma have been previously linked with reduced long term survival after STEMI[234]. That did not happen in the case of citH3-DNA[233].

In our cohort of patients, we found a higher association between Killip-Kimball score and plasma markers of NETs in patients of G1. Therefore, the time elapsed between the event and the sample collection can be crucial. It is worth noting that young ACS patients with the highest levels of citH3-DNA (> Q4), the most specific marker of NETs, had more frequently suffered a stroke before ACS than the rest of the patients.

Finally, we analyzed the occurrence of new adverse cardiovascular events. We found that patients from G2 with NET markers above the median (> Q2) in plasma and carrying the T allele of the SNP rs2431697 of miR-146a, had an elevated risk of recurrent ischemic events. Again, showing an important role of this variant in

thromboinflammation in young ACS patients, in consonance with our previous studies in other inflammatory pathologies[216,217,227].

In conclusion, the presented data show that in young patients, as it also happens in older ones, thromboinflammation is a pathologic state underlying ACS. Measuring NETosis components in plasma appears to be a useful marker of onset in these young patients. Besides, we described for the first time that the SNP rs2431697 could act as an additional risk factor for worse prognosis to ACS patients younger than 45 years. We hypothesize that rs2431697-T allele can establish a silent proinflammatory status that, when reaching the appropriate threshold, can predispose to an increased NETosis and, therefore, promote a higher rate of adverse cardiovascular events.

CHAPTER 2

STUDY OF THE INFLUENCE OF miRNAs IN THE EXPRESSION OF COAGULATION FACTORS INDUCED BY NETS COMPONENTS

The research presented below was published in the journal Biomedical Reports in November of 2018 [238]

1. INTRODUCTION AND BACKGROUND

As previously mentioned, immunothrombosis is a physiological process that links coagulation with innate immunity and its dysregulation, named thromboinflammation, may lead to the development of thrombotic events[119] being the NETs one of the main players of this process[154]. As detailed before (GENERAL INTRODUCTION: 2. Neutrophil extracellular traps), NETs are composed of DNA and carry diverse nuclear and granule-derived proteins, such as myeloperoxidase and neutrophil elastase (NE). But the predominant are histones, constituting around 70% of NET-binding proteins[239]. Those NETs components that lead to immunothrombosis are considered as DAMPs[125].

Since their discovery, several researchers have used the different NET components to investigate how NETs promote thrombosis. For instance, NE has been shown to inactivate the TFPI[240]. Isolated DNA is known to bind and activate contact system proteins, enhancing thrombin generation and clot formation in platelet-poor plasma[241,242]. DNA-free histones are highly cationic and, due to that, they can be cytotoxic for host cells[243]. They can promote thrombin generation in plasma by both platelet-dependent and independent mechanisms[244,245]. Thus, they are key in the induction of platelet aggregation, P-selectin expression and inhibition of protein C, all of them prothrombotic mechanisms described in ITP[246,247]. In this line, our group has described that NETosis is enhanced in ITP patients but independently of platelet activation[230], despite the fact that platelet proteins are significantly different in ITP patients compared with healthy individuals[248]. On the other hand, NETs boiled or denatured were not able to induce cytotoxicity, indicating that NET-mediated cytotoxicity is mostly due to proteins rather than to cell-cell interactions[243].

A more recent study has shown several previously unknown mechanisms of cytotoxicity by NETs components[249]. One important new function is that chromatin DNA regulates the cellular location of TLR4 and promotes TLR4 recruitment to endosomes containing chromatin. Also, cells may be able to regulate their sensitivity to those DAMPs thanks to the regulation of the cellular localization of TLR4. Finally, the chromatin fragmentation provides an extra layer of complexity for the regulation of those inflammatory responses. Enzymes that fragment chromatin without degradation

of nucleosome-bound DNA will trigger more inflammation by generating mononucleosomes, known to be pro-inflammatory. Enzymes that completely remove DNA will eliminate inflammation by suppressing the synergy between histones and DNA[250].

In this Chapter, we asked about the participation of miRNAs in the regulation of the expression of NET components which would promote a procoagulant state. In a previous work, we described the participation of miRNAs in the first phase of coagulation activation, as is the regulation of TF expression. Thus, we described the implication of the members of the miR-17/92 cluster and its paralog miR-106b-25 in modulating TF expression in the monocytic leukemia cell line (THP-1). The *in vitro* assays confirmed that miR-20a directly inhibits the expression of TF. Also, the inhibition of miR-20a led to a 50% increase of the procoagulant activity of THP-1 after induction with LPS. Furthermore, miR-19a was also shown to inhibit expression of TF in breast cancer cells[251].

An additional element that could be crucial in the thrombogenicity of NET components is HNF4α, a transcription factor essential for development due its key role in regulating procoagulant, anticoagulant, and fibrinolytic genes in the liver[252,253]. Our group demonstrated that miR-24 and miR-34a inhibit HNF4α both in HepG2 and healthy human hepatocytes. Therefore, these miRNAs might be able to regulate hepatic coagulation genes in humans via this hepatic factor[253].

The aim of this chapter was to investigate if the NETs components, histones, and DNA, induce a procoagulant state via overexpression of TF. In addition, we analyzed the role of these DAMPs on miRNAs that regulate the expression of TF.

2. MATERIALS AND METHODS

2.1. Neutrophils DNA purification

Whole blood was drawn in EDTA tubes for all healthy donors after providing written informed consent. Donors were unrelated Caucasian volunteers, enrolled in the local blood donor center (Centro Regional de Hemodonación, Murcia, Spain). The study was performed in accordance with the ethical standards of the Declaration of Helsinki and authorized by the Ethical Committee of the University Hospital Morales Meseguer, Murcia, Spain.

Neutrophils were purified using a gradient of Histopaque 1077 and 1119 (Sigma Aldrich; Merck KGaA, Darmstadt, Germany). Purity was checked by flow cytometry, considering the CD11b+/CD14- population as neutrophils. The purity was always > 90%. CD11b+ fluorescein isothiocyanate-labeled (cat. no. 11-0118-42) and CD14-phycoerythrin-conjugated antibodies (cat. no. MHCD1417; Thermo Fisher Scientific, Inc.) were used, applying 1 µL (0.05 µg) of antibody for $1x10^6$ cells in 100 µL of buffer [0.5% PBS-bovine serum albumin (Merck KGaA), 2 mM EDTA]. Measured in a BD Accuri™ C6 flow cytometer device (BD Biosciences, San Jose, CA, USA). DNA was purified with the DNeasy kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions.

2.2. Cell culture

The cell line HepG2 of human liver carcinoma (American Type Culture Collection (ATCC), Manassas, VA, USA) was cultured in Eagle's minimum essential medium (MEM) completed with the addition of non-essential amino acids at 0.1 mM and 10% of fetal calf serum (FCS) (Thermo Fisher Scientific, Inc.). The cell line of monocytic leukemia THP-1 (ATCC) was cultured in ATCC-formulated RPMI-1640 medium supplemented with 10% FCS (Thermo Fisher Scientific, Madrid, Spain) and both, THP-1 and HepG2, were maintained at 37ºC and 5% CO_2.

By quantitative reverse transcription-polymerase chain reaction (qRT-PCR), it was verified that *F3* (TF) mRNA expression was induced by DNA in a dose-dependent manner in THP-1 cells (0, 25 and 50 ng/µL) and the highest dose was used for subsequent experiments (**Figure 20**). THP-1 cells were activated in 24-well plates (500,000 cells/well) with 50 ng/µL of human neutrophil DNA or 20 µg/µL of human

recombinant histone H4 (New England BioLabs, Inc., Ipswich, MA, USA) following the indications previously published by Kim et al[254]. DNase/RNase free water was used as negative control and 1 µg/mL of LPS as positive control of cell activation. After 2 hours at 37ºC and 5% CO_2, THP-1 cells were harvested.

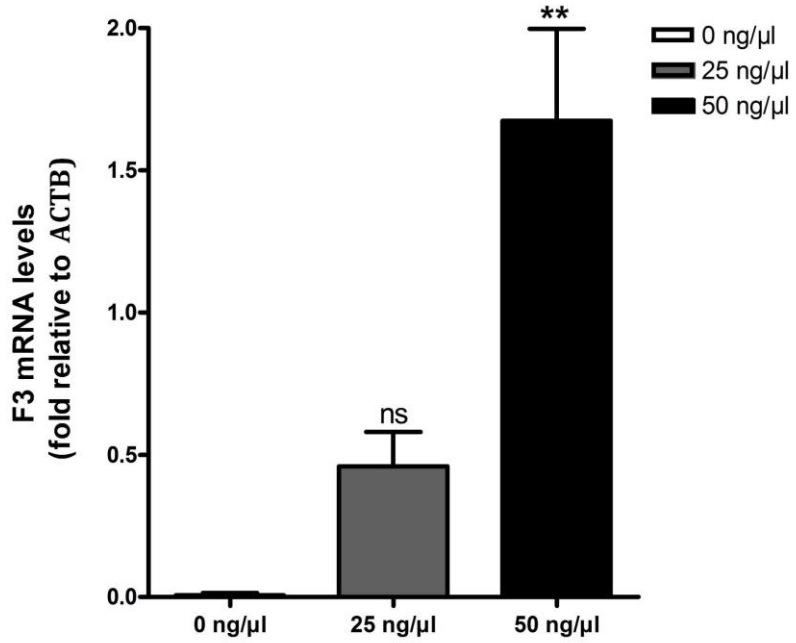


Figure 20. Dose-response of the activation of THP-1 cell line by DNA measured as mRNA levels of *F3* (Tissue Factor). ns: not significant; $^{**}p < 0.01$.

HepG2 cells were cultured in 12-well plates ($1x10^6$ cells/well) and stimulated with 50 ng/µL of DNA from human neutrophils or 20 µg/µL of human recombinant histone H4. The cells were harvested after 24 hours of incubation at 37ºC and 5% CO_2 .

2.3. RNA isolation and detection of mRNA expression by real-time qRT-PCR

Total RNA from the cell lines was isolated with RNAzol RT reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) following the manufacturer's protocol. MultiScribe Reverse Transcriptase and Premix Ex Taq Master Mix (Takara Biotechnology Co., Ltd., Dalian, China) were used with 100 ng of total RNA to perform the retrotranscription for both, mRNA and miRNA PCRs. qRT-PCR was performed using TaMan Gene Expression Assays (Applied Biosystems; Thermo Fisher Scientific) with

gene-specific primers (**Table 6**). The assays were performed using a Light-Cycler 480 Real-Time PCR system (Roche Applied Science, Penzberg, Germany). β-actin (*ACTB*) was used as endogenous reference control for normalization and quantification.

For the quantification of miRNA levels, TaqMan Gene Expression Assays were used with specific primers for the genes: hsa-miR-24, hsa-miR-17-5, hsa-miR-18a, hsa-miR-19a, hsa-miR20a and hsa-miR-106b. The endogenous control was U6 snRNA (Thermo Fisher Scientific, Inc.).

Table 6. List of gene specific qRT-PCR primers used in this study for protein coding genes.

Gene	Specific primer	Encoding protein
F3	Hs01076029_m1	Tissue factor (Factor III)
F5	Hs00914120_m1	Factor V
F7	Hs01551992_m1	Factor VII
F10	Hs00984443_m1	Factor X
F12	Hs00166821_m1	Factor XII
PROC	Hs00165584_m1	Protein C
PROS1	Hs00165590_m1	Protein S
SERPINC1	Hs00166654_m1	Serpin family C member 1
SERPINF2	Hs00168989_m1	Serpin family F member 2
HNF4A	Hs00230853_m1	Hepatocyte nuclear factor 4
ACTB	Hs99999903_m1	β-actin

2.4. Statistical analysis

All experiments were performed three times in triplicate. All data is presented as the mean ± SD. The non-parametric Mann-Whitney U test was used to assess statistical differences between groups, using GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA). We considered a p-value < 0.05 as statistically significant.

3. RESULTS

3.1. Histone H4 and DNA induce the expression of coagulation factors

First, the role of NET components on the expression of coagulation factors (listed in Table 6) in the human liver was analyzed. For that, HepG2 cells were incubated with DNA or histone H4 and 8 genes related to coagulation were quantified. Both components induced a significant elevation of the expression of both procoagulant (**Figure 21**) and anticoagulant factors (**Figure 22**). Histone H4 induced a slight but consistent greater increase than DNA when compared with the negative control corresponding to cells without treatment. In the case of *F10* and *PROC*, we found a trend toward the increase of expression, but without statistical significance (**Figure 21, C and Figure 22, C**). The *p*-values are specified in **Table 7**.

3.2. Histone H4 and DNA upregulate the expression of *HNF4A*

Next, *HNF4A* mRNA levels were quantified after incubation of HepG2 with histone H4 or DNA. Both NETs components upregulated its expression significantly and to a similar extent (DNA: $p = 0.028$; H4: $p = 0.007$) (**Figure 23**).

3.3. Histone H4 causes a decrease in the expression of miR-17/92 cluster

Finally, to confirm the influence of NETs on the increased expression of TF and the role of the miR-17/92 cluster in this process, THP-1 cells were incubated with either DNA or histone H4. After incubation the levels of *TF* mRNA and miR-17/92 cluster (miR-17-5, miR-18a, miR-19a, and miR-20a) and its paralog miR-106b, were measured. Both agonists, DNA and histone H4, significantly increased the expression levels of *TF* (DNA: $p = 0.007$; H4: $p = 0.0008$; positive control - LPS: $p = 0.005$) (**Figure 24 - A**). The levels of selected miRNAs did not change after incubation of THP-1 cells with DNA (**Figure 24 - B**), while all of them were significantly decreased when treated with histone H4 (**Figure 24 - C**). On the other hand, it is known that HNF4α is regulated by miR-24. Following the same hypothesis, miR-24 was measured after activation with histone H4 or DNA, but none of these DAMPs altered its expression. The *p*-values are specified in **Table 8**.

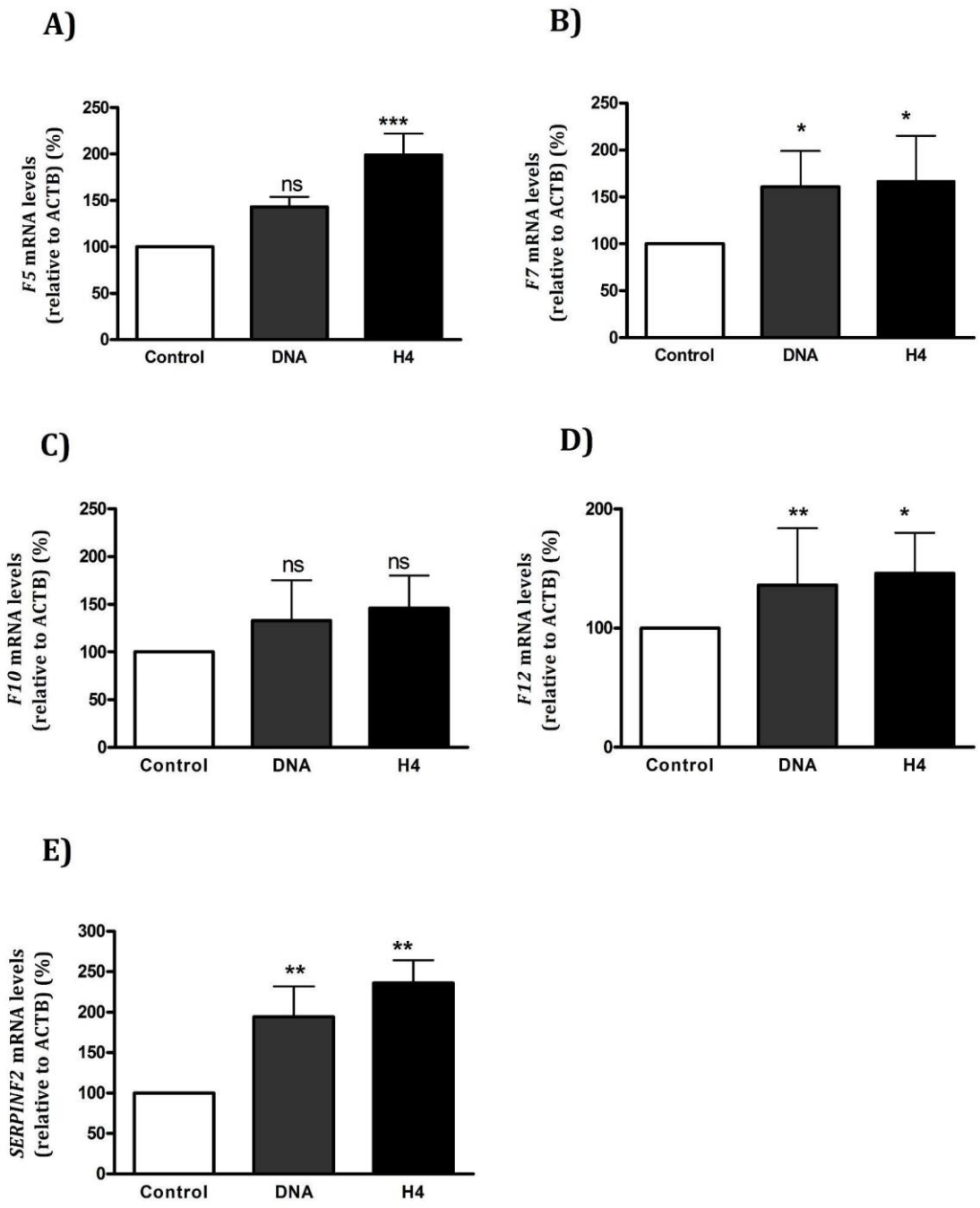


Figure 21. NETs components increased procoagulant factors in HepG2 cells. ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

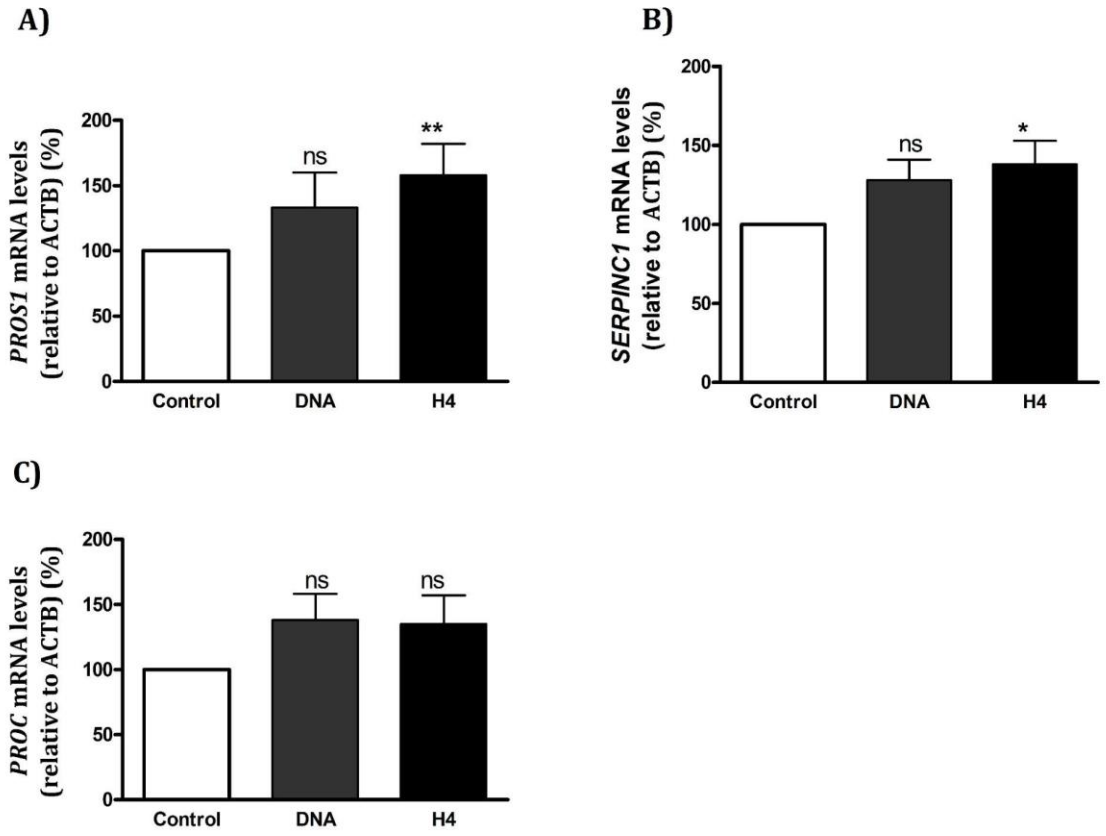


Figure 22. NETs components increased anticoagulant factors in HepG2 cells. ns: not significant; * $p < 0.05$; ** $p < 0.01$.

Table 7. List of *p*-values for the change in expression of each gene (compared to a non-activated control) with each agonist.

Procoagulant gene	**Activated with DNA**	**Activated with histone H4**
F5	$p = 0.053$	$p = 0.0003$ (***)
F7	$p = 0.031$ (*)	$p = 0.023$ (*)
F10	$p = 0.321$	$p = 0.110$
F12	$p = 0.003$ (**)	$p = 0.012$ (*)
SERPINF2	$p = 0.006$ (**)	$p = 0.004$ (**)
Anticoagulant gene	**Activated with DNA**	**Activated with histone H4**
PROS1	$p = 0.059$	$p = 0.003$ (**)
SERPINC1	$p = 0.081$	$p = 0.041$ (*)
PROC	$p = 0.200$	$p = 0.677$

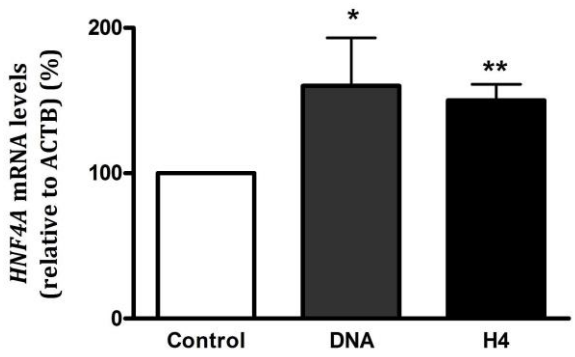


Figure 23. NETs components increased the expression levels of *HNF4A* mRNA in HepG2 cells. * $p < 0.05$; ** $p < 0.01$.

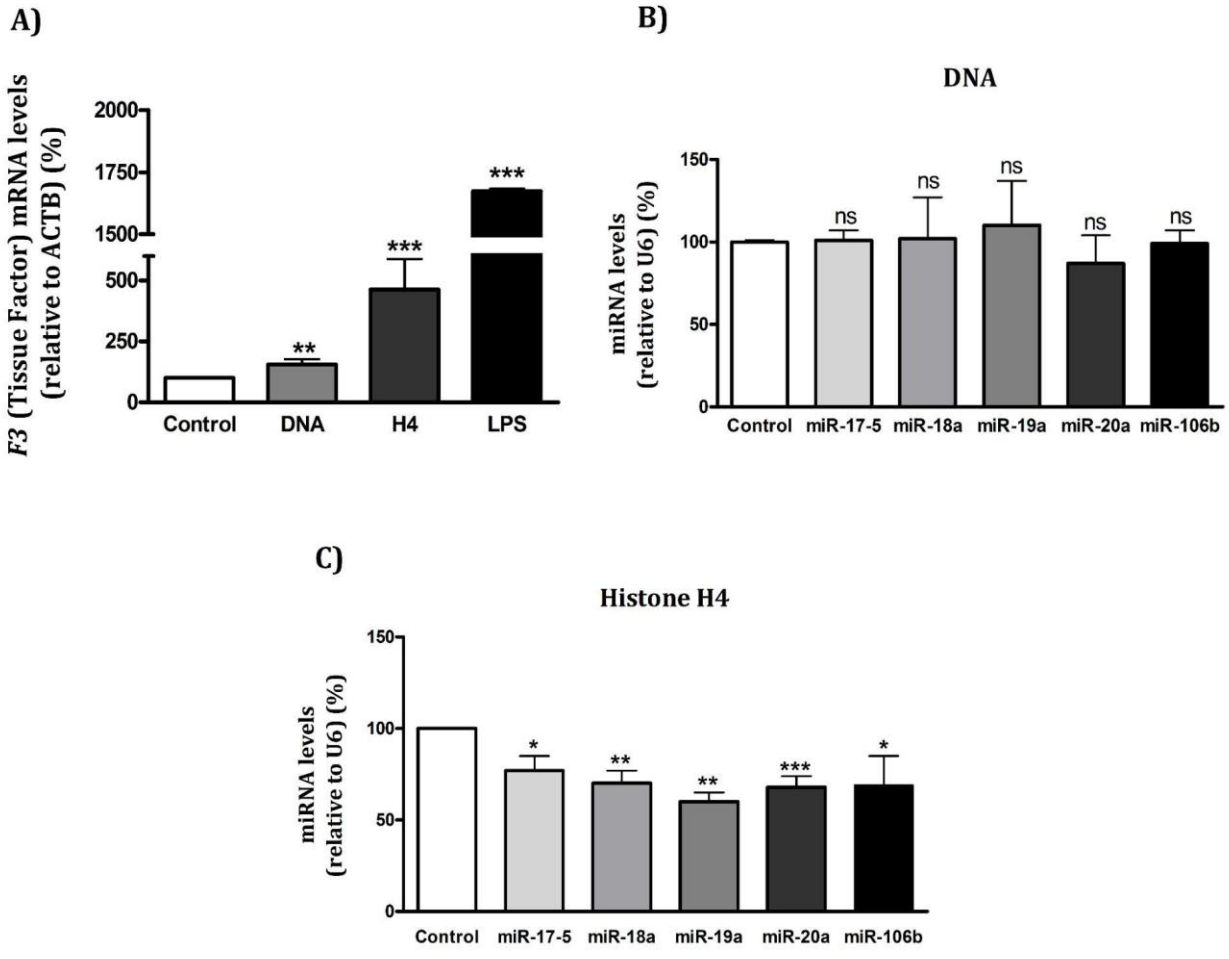


Figure 24. Expression of tissue factor (*F3*) and miR-17/92 in monocytic cells activated with DNA or histone H4 *in vitro.*

Table 8. List of *p*-values for the change in expression of miRNA (compared to a non-activated control) with each agonist.

miRNA	Activated with DNA	Activated with histone H4
miR-17-5	$p > 0.999$	$p = 0.036$ (*)
miR-18a	$p > 0.999$	$p = 0.001$ (**)
miR-19a	$p = 0.976$	$p = 0.002$ (**)
miR-20a	$p = 0.930$	$p = 0.0005$ (***)
miR-106b	$p > 0.999$	$p = 0.013$ (*)
miR-24	$p = 0.436$	$p = 0.667$

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

4. DISCUSSION

In the last decade, research regarding the pathogenicity of DAMPs and the prothrombotic role of DNA and histones has been reported[136,244,255]. Our results are in agreement with this hypothesis since we found that histone H4 and DNA induced the transcription of several coagulation factors. Specifically, the mRNA levels of *F7, F12* and *SERPINF2* (encoding α2-antiplasmin) were significantly upregulated by DNA and histone H4, while the mRNA levels of *F5, PROS1* and *SERPINC1* (encoding antithrombin) were upregulated only by histone H4.

Looking for a possible mechanism for this effect, HNF4α was selected as a suitable candidate. It has been demonstrated that this hepatic transcription factor is implicated in the transcription of numerous coagulation genes including *F5*, *F7*, *F12*, *SERPINF2* and *SERPINC1*[256–258] among others. In the current study, it was hypothesized that the observed increase in *HNF4A* expression may be responsible for the increase in the expression of certain coagulant factors induced by histone H4 and DNA, although a direct effect of these DAMPs on the overexpression of hemostatic factors cannot be discarded. Limited information is available concerning the mechanisms implicated in the action of histone H4 and DNA on different cells or tissues. It is noticeable that normal primary hepatocytes and monocytes would have been an optimal model to study the effect of NETs in the expression of coagulation factors, but due to the

variability between individuals that may difficult the interpretation of the results and the inaccessibility of hepatocytes, we decided to perform the experiments with cell lines that are well characterized and allow to obtain homogeneous results.

To date, one of the sole proposed mechanisms implicated in TF expression induced by histones in monocytes is the activation of the TLR2-4/NF-κB pathway. In that report, it was demonstrated that the selective blockade of TLR2 or TLR4 inhibited the overexpression of TF following activation with histone H4, among other histones[254]. More recently, it has also been shown that citrullination can trigger the binding of nucleosomes to TLR4[249]. Notably, the expression of TLR4 has been reported in hepatocytes and has been proposed as interesting to investigate if its molecular pathway may be implicated in the overexpression of hemostatic factors and/or HNF4α as for TF[259]. Additional studies using animal models will aid to define if these changes in the coagulant state does actually occur *in vivo* and if overexpression of hemostatic factors is also detectable in plasma following a challenge with DNA or histone H4.

miRNAs have been previously associated with the regulation of several hemostatic factors[260], in particular, coagulation factors including fibrinogen, factor XI and TFPI[182,261,262]. Another key element, TF has also been demonstrated to be regulated by miRNAs[260]. Thus, in the present study it was hypothesized that a possible explanation for the increased expression of TF by DAMPs in monocytes may be an alteration of miRNA expression. In this regard, it was identified that, following the treatment with histone H4, the decrease of miRNAs targeting TF was significant. It has been demonstrated that activation of TLR2 in synoviocytes by bacterial lipoproteins or of TLR4 by LPS decreased the expression of miR-19a/b and miR-20a, respectively[263,264]. These results are in concordance with the present findings. Yet, the mechanism by which these miRNAs are decreased by DAMPs is still unknown. In opposition, DNA had no effect on the expression of miRNAs in the miR-17/92 cluster, suggesting that the upregulation of TF by DNA is independent of miR-17/92 and other unknown mechanisms are involved.

In conclusion, the present results indicated that histone H4 and to a lesser extent, DNA, cause an increase in the mRNA expression of several coagulation factors. This upregulation may alter the hemostatic balance and may explain the prothrombotic

effect of histones and DNA, though this hypothesis requires further confirmation. Additionally, both DNA and histone H4 triggered an increase in *HNF4A* mRNA expression, which may indicate its partial involvement in the regulation of clotting factors mediated by DAMPs. Finally, our results suggest that some miRNAs could be involved in the prothrombotic effect of these DAMPs. Thus, although we did not find an increase in the expression of miR-24, which we had previously demonstrated to be related to HNF4α[253] it was determined that histone H4 could induce a decrease in the expression of certain miRNAs of the miR-17/92 cluster. These findings may partially explain the upregulation of the expression of TF induced by histone H4, while DNA would act independently of miR-17/92.

CHAPTER 3

SEARCH FOR miRNA VARIANTS IMPLICATED IN ACUTE CORONARY SYNDROME IN PATIENTS <45 YEARS

1. INTRODUCTION AND BACKGROUND

After decades of being considered "junk genome", the non-coding RNAs are now considered as key players in a multitude of physiologic and pathologic processes. Specifically, the best known and most popular non-coding RNAs are miRNAs, further described above (GENERAL INTRODUCTION: 3. MicroRNAs). Due to the crucial regulatory role of miRNAs, an alteration in their expression or function may be critical for the related biological processes and can trigger human diseases. Moreover, a genetic change in the mRNA target can also have pathologic consequences. Thus, a single base mutation in the 3'UTR of the target site for miR-189 in the gene SLITRK1 has been associated with Tourette's syndrome[265]. Similarly, Mencía et al. described in 2009 the first pathogenic mutation in the sequence of a mature miRNA, a single nucleotide change in the seed region of miR-96 resulting in progressive hearing loss[266]. In 2014, two mutations were found in the seed region of miR-142-3p. These mutations induced the redirection of the miRNA to target the transcriptional repressors the *ZEB2* and *ZEB1* mRNAs, as well as the loss of regulation of its already known target, *RAC1*, coding for the Rac family small GTPase 1, and *ADCY9,* coding for the Adenylate cyclase 9 protein. These mutations have been reported in 19.6% of diffuse large B-cell lymphoma patients[267]. Finally, the most recently described pathological mutation in the sequence of a miRNA is a gain-of-function mutation in miR-140 resulting in a novel autosomal dominant skeletal dysplasia. The mutation causes a high miR-140-5p expression of the mutated form, therefore, eliminating the repression of targets of the WT miR-140-5p and in turn, increasing the repression of new targets[268].

In the cardiovascular field, it is well known that miRNAs are plentifully present in several cardiac cell types and there is even a "subclass" of miRNAs called MyomiRs that are fundamental for cardiovascular development[269]. Many studies on preclinical models have demonstrated that targeting miRNAs would represent a bright therapeutic tool for cardiovascular diseases. Looking in the National Institute of Health website: https://clinicaltrials.gov, nowadays we can find 223 clinical trials under "cardiovascular diseases and miRNA" premises. This is due to the well established function of miRNAs in diverse cardiovascular diseases. For instance, changes in miRNA expression levels have been widely implicated in cardiac hypertrophy[270]. Thus, miR-101 has been described to be significantly downregulated in hypertrophic hearts and also in post-infarcted ones, described therefore as an antifibrotic miRNA[271]. For further examples, miR-21 and miR-

155 also have relevant roles in cardiac hypertrophy, fibrosis, and remodeling[272,273]. Likewise, the ischemia/reperfusion injury is remarkably regulated by miRNAs. Most of them target, and negatively regulate, BCL2, an anti-apoptotic gene[274–276]. Lastly, several miRNAs have been involved in the pathophysiology of atherosclerosis, regulating processes such as lipid uptake, recruitment of inflammatory mediators, cellular adhesion, and so on[277–279]. As mentioned in CHAPTER 1, miR-146a has been linked with the pathophysiology of atherosclerosis[218,280,281].

For studying miRNA profiling, there are currently three well-established approaches: qRT-PCR, hybridization methods and high-throughput sequencing. The first one, qRT-PCR consists of a reverse transcription of miRNA to cDNA and a quantitative PCR with real-time monitoring (real time PCR). To scale it for hundreds of miRNAs, several medium-throughput platforms offer pre-plated PCR primers in 348 wells plates or microfluidics cards. The hybridization-based methods are best known as microarrays. They were one of the first methods used to analyze a large number of miRNAs. Microarrays are less expensive, but have the inconvenience of imperfect specificity for miRNAs with closely similar sequences, their best use is for comparing specific miRNAs relative abundance. Finally, RNA-seq is a next generation sequencing (NGS) technique that, along with the relative quantification of miRNA expression, also gives the precise miRNA sequence[282] (**Figure 25**).

Patients that have suffered ACS before 45 years have a relatively low percentage of classical risk factors compared with older patients and the role of inheritance is greater, so we selected this young cohort to be able to study the genetic component of the disease. Due to the demonstrated importance of miRNA mutations in the onset of different pathologies, our hypothesis supports that new variants in genes of miRNAs related to inflammatory and atherothrombotic processes may be associated with the onset and outcome of ACS. Therefore, we needed to rely on NGS technology to find new mutations related to ACS. NGS platforms allow paired-end sequencing, making it possible to read both ends of the same DNA fragment. This sequencing strategy facilitates not only the positioning of those reads that can map to multiple sites, but also the identification of structural variants[283].

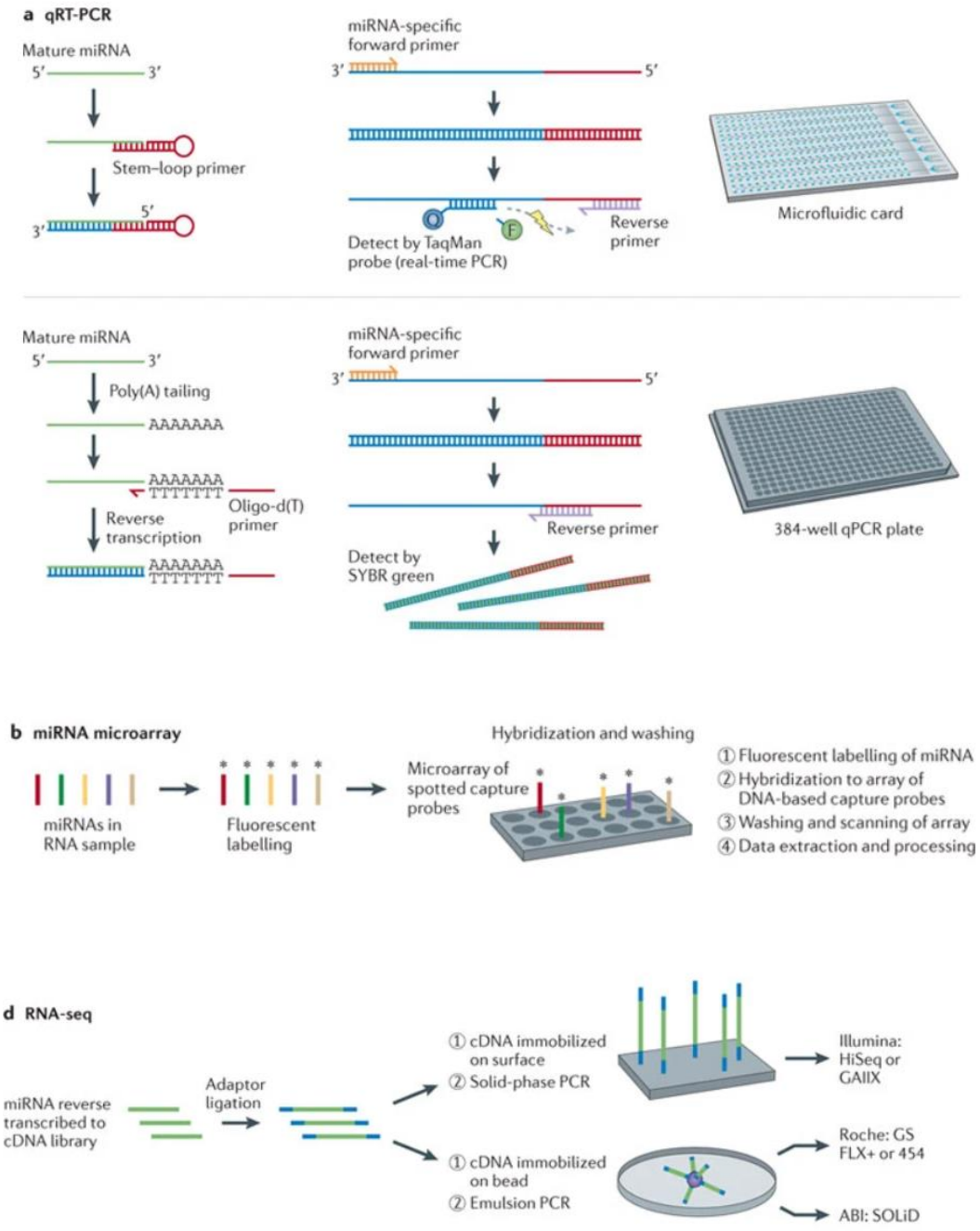


Figure 25. Representation of the three most used approaches to miRNA profiling: qRT-PCR, miRNA microarrays and RNA-seq (NGS technique). *(Adapted from Pritchard et al., Nature Reviews Genetics, 2012)*[282]

This chapter builds on a previous work in which we performed an NGS in 150 young ACS patients and 150 healthy donors for selected 365 miRNAs. The selection of these miRNAs was based on their expression in one or more of cell types or tissues potentially involved in ACS: platelets, leukocytes, cardiomyocytes, endothelial cells and

liver tissues. By using the Ion PGM system of Ion Torrent (Life Technologies) we sequenced patients and donors (the later were paired in age and sex with the patient cohort). Grouping the individual samples in 5-sample pools, given that it has been previously described as a robust and cost-effective method for rare variants discovery and showed the same accuracy as individual sequencing[284]. Therefore, we sequenced 30 pools of patients samples and 30 pools of healthy donors samples. In those 365 miRNAs, we found 1175 variants in miRNA genes after applying the established quality tests, Fisher's test and a binomial test to ensure that the variants found are trustworthy. Based on these results, we searched for miRNA variants potentially involved in ACS in patients below 45 years.

2. STUDY POPULATION

2.1. NGS patients and donors

To perform the NGS, 150 patients that suffered their first ACS episode before the 45 years of age were enrolled between January 2015 and December 2016. Subsequently, 150 healthy donors from the local blood donation center (Centro Regional de Hemodonación) were recruited matching as much as possible in age and sex with ACS patients. The population was mainly composed of males (ACS: n= 130, 87%; healthy individuals: n= 114, 76%) and the median age was 42 years in both groups (range: 22 - 45) **(Table 9)**.

2.2. Enlargement of the cohort for genotyping of the selected variants

To study the frequency of the selected variants, we extended the patient cohort with a new set of 150 patients (making a total n= 300), enrolled between January 2017 and March 2020, and 150 new healthy donors (making a total n = 300), again matching both populations in age and sex. Both populations were mostly males (ACS: n= 263, 87%; healthy individuals: n= 251, 84%) with a median age of 44 years for ACS patients and 42 for healthy donors, both within the same range (22 - 45) **(Table 9)**.

2.3. General population cohort

Finally, an additional set of 300 healthy donors were enrolled during the blood donation campaigns of our local blood transfusion center during 2019. This donor cohort was no longer matched with the patient's characteristics to be a representative sample of the general population. There were still more males than females, but in a more similar percentage than in the previously recruited (n= 175, 58% *vs.* n= 125, 42%). All of them were within the range of age required to donate blood (18 - 64), with a median age of 42 years (**Table 9**). Written informed consents were provided for all individuals and the study was authorized by the Ethics Committee of Hospital Clínico Universitario Virgen de la Arrixaca and Hospital General Universitario Morales Meseguer in Murcia, Spain.

Table 9. Age and sex of the ACS cohort *vs* healthy donors in the different groups.

		Healthy donors (n = 150)	**ACS Patients (n = 150)**
NGS cohort	Age, mean (range)	42 (22-45)	42 (22-45)
	Male sex, N (%)	114 (76)	130 (87)
		Healthy donors (n = 300) *including NGS cohort*	**ACS Patients (n = 300)** *including NGS cohort*
Genotyping cohort	Age, mean (range)	42 (22-45)	44 (22-45)
	Male sex, N (%)	251 (84)	263 (87)
		Healthy donors (n = 300)	
General population	Age, mean (range)	42 (18-64)	
	Male sex, N (%)	175 (58)	

3. MATERIALS AND METHODS

3.1. Genomic DNA extraction

Blood was drawn in EDTA tubes for all patients and donors. These samples were centrifuged at 2500x g during 15 minutes at RT within 24 h after collection. The buffy coat was used for DNA extraction using the QIAcube device and QIAamp DNA Blood Mini Kit (Quiagen, Madrid, Spain), following the manufacturer instructions. DNA samples were stored until their analysis at -20ºC.

3.2. Selection of potentially pathological variants

To be considered, the variant had to be supported by a high number of reads and present in a non-conflicting region of the genome. For this first screening we used the Integrative Genomics Viewer (IGV). The management of the results of the NGS was carried out in collaboration with the Bioinformatics Platform from the IMIB-Arrixaca. As a result of this collaboration, a tool for filtering the variants was created, which significantly facilitates the selection. Several filters were generated, such as the statistical tests, the quality of the reads, the coverage, population, miRNA gene, etc.

The following criteria were used to select variants (**Figure 27**):

1. We seeked for those variants present only in patient pools and in no control pools, or in the case of being present in some control pool, the ratio control:patient must be at least 1:4.
2. Then we selected only those variants with a reported minor allele frequency (MAF) under the threshold of 0.05.
3. The final consideration to select the variants was their relationship with cardiovascular diseases or cardiovascular-related pathways via bibliographical research.

3.3. Genotyping of selected variants

The final 10 selected variants were genotyped in the individual samples using custom primers with KASP (*kompetitive* allele specific PCR) technology (LGC Genomics)[285,286], an uniplex single nucleotide variant (SNV) genotyping platform. Following the recommended protocol, we used 2.5 µL of KASP Master mix, 0.07 µL of KASP assay custom mix, 0.03 µL of MgCl2, 0.4 µL of ultrapure water and 2 µL of DNA (5 µg) per sample. The recommended thermal cycling conditions are specified by the manufacturer for each specific probe (**Table 10**). Finally, the assay was performed in a LightCycler 480 (Roche Farma, Madrid, Spain).

Table 10. KASP genotyping information for each custom probe.

Assay ID	FAM allele	HEX allele	Sequence	Recommended thermal cycling protocol
rs9589207	G	A	CTGTAGAAAAGTAAGGGAAACTCAAACCCCTTTCTACA CAGGTTGGGATC[G/A]GTTGCAATGCTGTGTTTCTGTA TGGTATTGCACTTGTCCCGGCCTGTTGA	61-55°C Touchdown protocol
rs186354597	G	A	AGATCTCACTTCCCCACAGAAGCTCTTGGCCTGGCCTCC TGCAGTGCCAC[G/A]CTCCGTGTATTTGACAAGCTGAG TTGGACACTCCATGTGGTAGAGTGTCA	61-55°C Touchdown protocol
rs117258475	G	A	CTCCTGGCACACAGCTGCGGGTGCAGCGCTCTTCAGAG CCCTTCAGGAGA[G/A]CCTCCACCCAACCCTCTGAATT AGGCACAACAGGATTGAGGGGGGGCCCT	61-55°C Touchdown protocol
rs114332141	A	T	CCAACCATGTTCTGACCACTGCCTGGGGGGCTTCTCGG AGGTAGAGAGAG[A/T]GAGAGAGCAAGAGGGGAGAGA GCGAGCCAGCAAGCCAGCGAGCCTGCAGC	68-62°C Touchdown protocol
rs374297906	G	A	GGTGCTCCAGAGCAGAGGAGAGGTGTCCAGCGGTGGTG GCGGCGGTGGCGG[G/A]CCATGCTGCAGGAGAGCACGG TGCTTTCCGCGGTG	Failed validation - unable to genotype
rs142673707	T	A	CTCCCCTGGGGTGAGCCAACTTACCTGATGCTTTTAGG CTTAATTGAGCA[T/A]AAGTTTGATTGGCAGCGTCTGC CCGGCCCTGCCCTCACGCCCTGACCCTC	68-62°C Touchdown protocol
rs41279088	G	A	TGGGGTTGGCTCGCCCCCTCTCAAGAGACCTCACCTGGC CTGTGGCCAGG[G/A]TCCCCTGTAGCAACTGGTGAGCG CGCACCGTAGTTCTCTGTCGGCCGGCC	61-55°C Touchdown protocol
rs557283175	C	T	GGTGAGGGAGGCTCTTTGGGCAGACCGGCGGCTGCCCA CATCCCCAGTCC[C/T]CATTGACCCCCCCATCCTCTCAG TCTTCCTCGGCCACCCTTCCCCCATCA	68-62°C Touchdown protocol
rs9621867	A	G	TTTCCCCCCGGGCAGAGGCCCCGCCCCAGCCAGCCTGCA TTCCAGGTCTC[A/G]GATCCCTGCAGACCACCCTGGGG GAGGCACTGGCAGGCCTGCCCGACACT	68-62°C Touchdown protocol
rs1007569411	T	C	TAAGAGATGGGCCAGTGGTGCTCCCAGGGTCTGCACCT CGACAGTCCAGG[T/C]GGCAAGTCTGATTTCGGGTGCC CCGCAATCAACGCCGAATACTCCA	61-55°C Touchdown protocol

3.4. Validation by Sanger sequencing

To validate the two final selected variants found by NGS, we performed Sanger sequencing in all the SNV-carrier samples. Specific primers for the genomic region of each variant were designed using Primer3Plus[287]. We performed the pre-sequencing PCR using Kapa2G (Roche) with these primers (**Table 11**). Subsequently, the PCR product was purified using ExoSap-ITTM (Applied Biosystems) and Sanger sequencing was performed on a 3130xl Genetic Analyzer (Applied Biosystems). The results were analyzed in Sequencing Analysis Software version 6 (Applied Biosystems) and the sequences were aligned using annotations of the reference genome GRCh38/hg38.

Table 11. List of primers used to perform Sanger sequencing.

Variant	Primer	Gene
rs117258475 - Forward	ATCGACTGTCCTGTCTCCACT	miR-296
rs117258475 - Reverse	AATTGTGAAATCAGGCCCAGC	miR-296
rs557283175 - Forward	CTTGCACTAGGACCACTGGG	miR-199b
rs557283175 - Reverse	GTTAGGCTGGGCTGGGTTAG	miR-199b

3.5. CRISPR-Cas9 functional studies

To analyze the functionality of the selected variants we rely on CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and Cas9 (CRISPR-associated) endonucleases. With that aim, I did an international stay at the Dr. Di Paola lab (Washington University School of Medicine in St. Louis (WUSTL), Missouri, USA), aiming to create a CRISPR-Cas9 knock-out (KO) cell model of the whole miRNA gene where the variant is located and a knock-in (KI) model with the specific SNV. Because of its location within the mature region of miR-296, our first choice to start this assay was rs117258475. For this study, due to the extra difficulty to modify miRNA gene regions, we ought to perform several different strategies optimizing the CRISPR assay. Given that, at the present time, the experiments are ongoing, those strategies are fully explained in the **Appendix**.

3.7. Statistical analysis

Pearson chi-squared test was used when comparison of proportions was needed. Mann-Whitney U test or Student t-test were used for assessed differences between continuous and categorical variables, as appropriate. We considered a *p*-value < 0.05 as statistically significant. Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software Inc.).

4. RESULTS

4.1. Selection of 10 potentially pathological miRNA variants

From the total of 1175 variants, found after performing NGS in the selected 365 miRNA genes, we selected 154 that met the selection criteria described in the Methods section. To note that the majority of those variants were present in 1 patient pool and no control pools (**Figure 26**).

Among those 154 variants, only 63 had a known MAF and 54 had a MAF below 0.05, our established threshold. Finally, for those 54 miRNAs, we performed a bibliographical analysis looking for a relationship with cardiovascular related pathways. We restricted our final selection to 10 variants (**Figure 27** and **Table 12**).

4.2. Study of the frequency in young ACS patients and healthy individuals

As detailed before, to validate and study the frequency of the selected variants, we extended the ACS and the healthy donor cohorts with 150 new individuals in each group (**Table 9** and **Figure 27**). We genotyped the 300 ACS patients and 300 healthy controls for each one of our 10 previously selected variants.

Two of the variants found by NGS were not validated by KASP genotyping. Rs114332141 and rs142673707, were present in 2 different pools each in the NGS, but KASP genotyping showed that none of the patients were actually carriers of these variants. They were probably sequencing artifacts. All other variants were validated with the KASP genotyping. The frequency found for each of the 10 selected variants is described in **Table 13**.

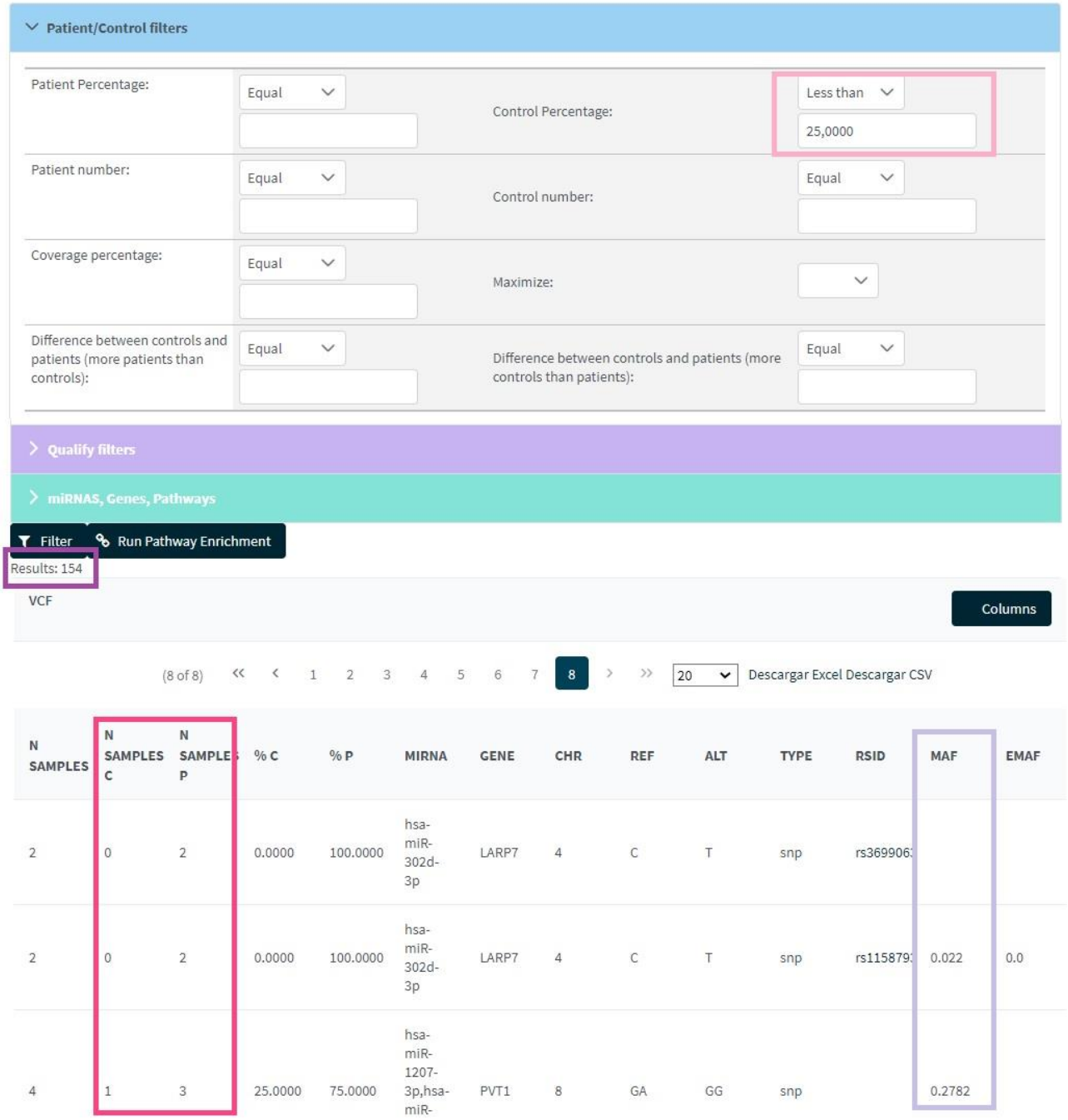


Figure 26. *In silico* tool for simplifying the selection of variants among all the NGS results. From top to bottom: Marked in light pink, the selective filter determining the number of controls permitted. In purple, the number of variants that fulfill the criteria. In dark pink the number of pools that carry each variant. Finally, in light purple, the MAF, that will be also used as another criterion later on. N SAMPLES C: number of samples from controls. N SAMPLES P: number of samples from patients. MAF: Minor allele frequency.

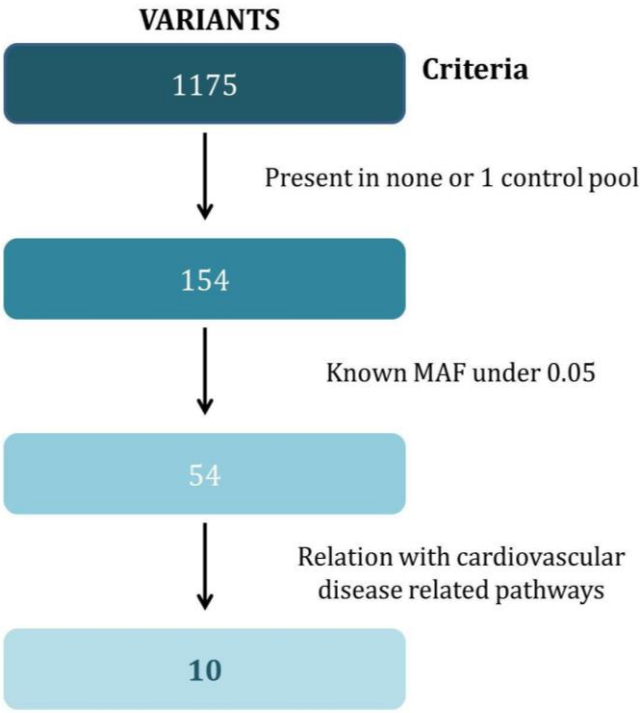


Figure 27. Number of variants found in each step of the selection.

4.3. Validation by Sanger sequencing

For the remainder of the study, we focused on the 2 variants significantly different between the disease and healthy populations, rs117258475 and rs557283175. To validate the results of KASP genotyping, we performed Sanger sequencing for each of the 12 carriers of rs117258475 and the 5 carriers of rs557283175 and each one of the carriers was confirmed (**Figure 28**).

Table 12. Description of selected potentially pathological SNVs and the references related to cardiovascular disease.

		NGS cohort		
SNV	**miRNA**	**Control pools n= 30**	**Patient pools n= 30**	**References**
rs9589207	miR-29c	0	1	288–291
rs186354597	miR-223-3p/5p	0	1	292–294
rs117258475	miR-296-3p	1	5	295–297
rs114332141	miR-377-3p	0	2	298–301
rs374297906	miR-218-2-3p	0	2	302–304
rs142673707	miR-495-3p	0	2	305–308
rs41279088	miR-141-3p/5p	0	2	309–312
rs557283175	miR-199b-3p/5p	0	3	313–315
rs9621867	miR-130b-3p	1	4	316–318
rs1007569411	miR-29c	0	2	288–291

4.4. General population frequency

To confirm the normal frequency of both variants, we collected samples from a general population following the restriction established for an ordinary blood donation (being between 18 and 64 years old, weight more than 50 Kg, and being generally healthy). Therefore, 300 subjects, males and females, between 18 and 64 years old were included in this study (**table 9**). We found one carrier for each SNP in the general population, a frequency of 0.0034 for both variants. This frequency is almost 4-fold smaller than the EMAF described in the case of rs117258475 and 30-fold higher for rs557283175 (**table 14**). These frequencies in the general population emphasize the high frequency of these variants in the ACS cohort (**table 15**).

Table 13. List of the SNVs, the miRNA gene where they are located and their frequency in healthy individuals and ACS patients. Marked in green the SNVs that are significantly different (chi-square) between young ACS patients and healthy donors. In gray, the SNV that was not genotyped for technical reasons.

		KASP genotyping			
SNV	**miRNA**	**Controls (frequency) n= 300**	**Patients (frequency) n= 300**	***p*-value**	**EMAF**
rs9589207	miR-29c	0	1 (0.0034)	0.317	0.0002
rs186354597	miR-223-3p/5p	2 (0.0067)	4 (0.0133)	0.412	0.0045
rs117258475	miR-296-3p	1 (0.0034)	11 (0.0367)	0.004	0.0126
rs114332141	miR-377-3p	0	0	-	0.0004
rs374297906	miR-218-2-3p	Unable for genotyping		-	0.0026
rs142673707	miR-495-3p	0	0	-	0.0041
rs41279088	miR-141-3p/5p	0	2 (0.0067)	0.157	0.0022
rs557283175	miR-199b-3p/5p	0	5 (0.0167)	0.025	0.0001
rs9621867	miR-130b-3p	2 (0.0067)	6 (0.002)	0.155	0.0004
rs1007569411	miR-29c	0	2 (0.0067)	0.157	0.00001

SNV: Single nucleotide variant. EMAF: European minor allele frequency. Chi-squared *p*-value.

4.5. Functional validation of the selected variants

Given the significant differences in the frequencies found for rs117258475 and rs557283175 among patients and controls, we aimed to study their functionality and evaluate their influence in the outcome of ACS using CRISPR-Cas9 technology. At the time of writing this Thesis, the results of this section were still pending. The preliminary results can be found in the **Appendix**.

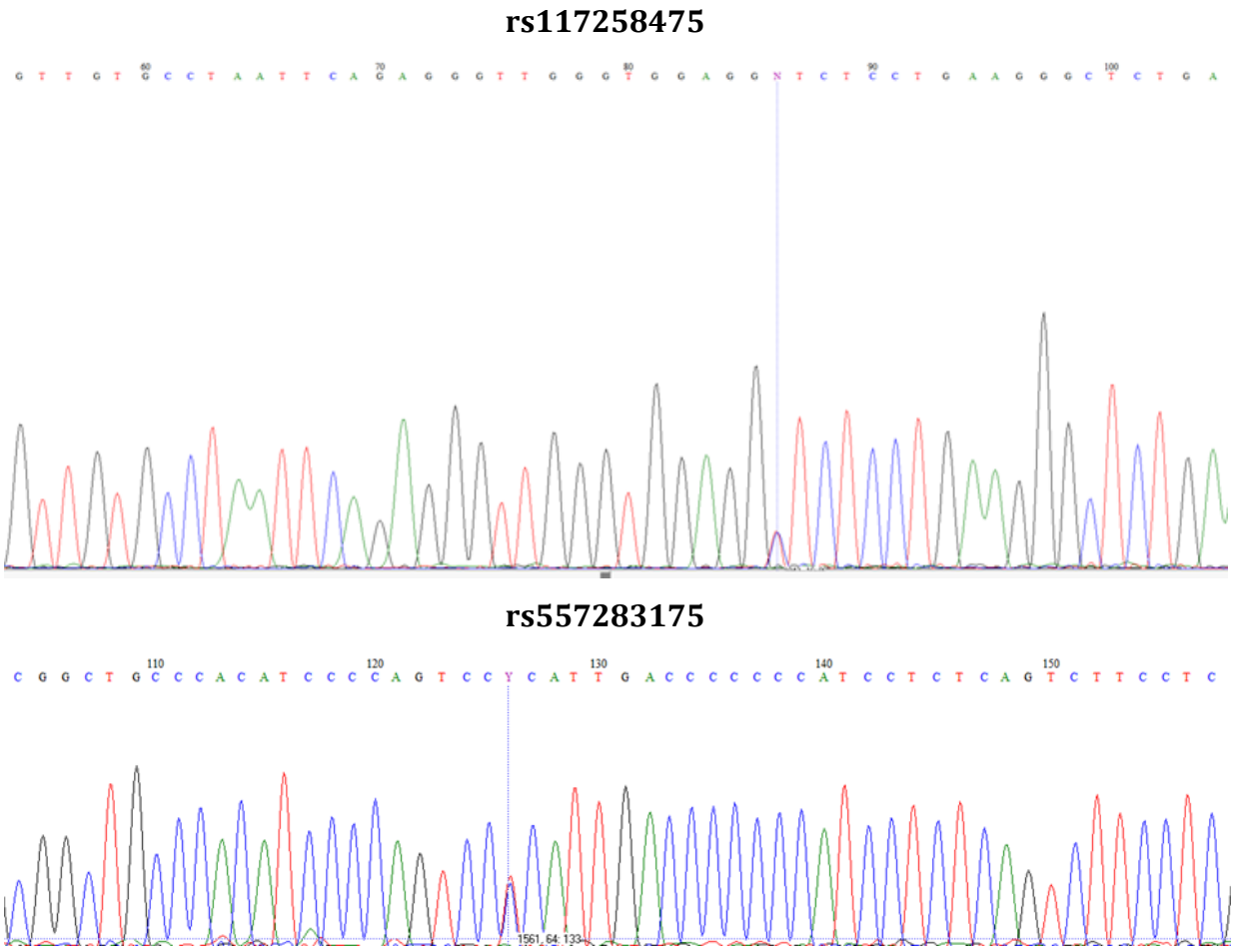


Figure 28. Representative images of Sanger genotyping for rs117258475 and rs557283175 heterozygous carriers.

Table 14. Frequency of the selected variants in a representative sample of the general population.

		General population	
SNV	**miRNA**	**Healthy individuals (frequency) n= 300**	**EMAF**
rs117258475	miR-296-3p	1 (0.0034)	0.01259
rs557283175	miR-199b-3p/5p	1 (0.0034)	0.0001

SNV: Single nucleotide variant. EMAF: European minor allele frequency.

Table 15. Frequency of the selected variants in all the study population, 600 healthy donors and 300 ACS young patients.

All study population					
SNV	**miRNA**	**Healthy (frequency) n= 600**	**ACS (frequency) n= 300**	***p*-value**	**EMAF**
rs117258475	miR-296-3p	2 (0.0034)	11 (0.0367)	< 0.0001	0.01259
rs557283175	miR-199b-3p/5p	1 (0.0017)	5 (0.0167)	0.029	0.0001

SNV: Single nucleotide variant. EMAF: European minor allele frequency. Chi-squared *p*-value.

5. DISCUSSION

When ACS occurs early in life, the role of classical risk factors is normally smaller than the inheritance component[47]. Our approach focuses on the search of genetic alterations affecting miRNA genes that associate with ACS onset and/or recurrence. So far, pathological mutations have been attributed almost fully to protein coding genes; nowadays we have already known that a greater amount of transcriptional control is due to non-coding RNA[319] and mutations in miRNA genes are beginning to be associated with human diseases[267,268].

This research aims to expand the knowledge about the miRNA genes involved in inflammatory and atherothrombotic processes leading to ACS and uncover pathological variants in those genes that may become a key prognostic marker or even a therapeutic target. Normally, miRNA regions have a low density of SNVs, suggesting the functional importance of conservation of miRNA sequences. Therefore, changes in these regions will most likely contribute to disease susceptibility[320].

After finding a total of 1175 variants located among 365 miRNA genes, we narrowed it down to 10 variants, after several filters. First of all, we searched for those variants present in none control pools in the NGS or, in case of being present in control pools, with a ratio of 1:4 with the patients pools, since our aim is to find very infrequent variants. We did not find any variant present in that proportion in more than 1 control pool. We also seeked only for those variants with a known MAF, but under 0.05. The last

filter being that the selected variants had to be located in miRNAs with a previously reported relationship with cardiovascular diseases or cardiovascular-related pathways. We were technically able to study the frequency of 9 out of that 10 variants in a 300 young ACS patients population and 300 matched controls, finding 2 of them to be significantly more frequent in ACS patients than in healthy individuals.

Interestingly, the variant rs117258475 is located in the mature region of miR-296-3p, only 4 base-pair (bp) apart from the seed region and this miRNA has been linked with a wide variety of important pathways. It has been related to the inflammatory response through macrophage polarization[198,321], NF-κB and IKBKE pathways regulation[297,322] and regulation of leukocytes and monocytes adhesion molecules[323,324], which play a key role in arteriosclerosis development. The cholesterol metabolism regulator multidrug resistance protein 1 (MDR1)[325,326] and the hypertension-causing gene WNK4 (with-no-lysine kinase 4)[327] are also miR-296-3p targets. In addition, it has been described that hypertensive patients have lower expression of miR-296 than healthy controls[328]. It should be noted that miR-296 is considered as an "angio-miR" since it has been associated with angiogenesis[296]. In the present work, rs117258475 variant was found in 3.7% of the ACS patients and in 0.3% of the healthy donors, which is significantly different among both populations and suggests an association with the disease that deserves further experimentation. On the other hand, the described European frequency was 1.3%, and was different from the frequency found in our healthy cohort.

On the other hand, rs557283175 is located up-stream of the mature region for miR-199b and has been less extensively studied in relation to the cardiovascular system than miR-296. But it is still important in that context, since it has been very recently associated with type 2 diabetes[329] and it is involved in endothelial cells differentiation from induced pluripotent stem cells by targeting the Notch ligand JAG1[314]. Furthermore, in a mice model of myocardial infarction, this miRNA was found elevated in the post-infarction heart and its silencing ameliorated cardiac dysfunction[330]. Indeed, it has been proposed as a therapeutic target in heart failure[331]. Also, close to this variant, but around 200 bp up-stream miR-199b, there is another miRNA, miR-3154. So far, this miRNA is not well studied, being related mostly to cancer and, in any case, to cardiovascular diseases. The variant is also located inside an intron of the coding sequence for Dynamin-1. This protein part of a large GTPase family required for

membrane fission, it was classically considered to be specific of neurons, but it has been described it presence upon activation in some non-neuronal cells, such as non-small lung cancer cells[332,333]. The only relationship of Dynamin-1 with cardiovascular pathologies was proposed as a result from a microarray expression profiling where it has been found to be a potential regulator for blood pressure regulation[334].

In our study, this variant was found significantly overrepresented in ACS, i.e. in 1.7% of the patients and in 0.2% of healthy donors, the European described frequency is 0.01%. These differences, in both SNVs, between the described frequency and the frequency in our cohort may be explained by the difference between populations in genetic frequencies, the cohort used in this study is European, but composed mostly of only Spanish individuals, and concretely from Murcia region. Also, the population used to calculate the EMAF almost doubles the size of our study cohort.

The lack of functional data from both variants does not allow the extrapolation of our results to the pathophysiological field. Even so, the frequencies found in our cohorts strongly suggest a link between them and the ACS onset and are sufficient to state the utility of these SNVs as new biomarkers of the disease with potential functional effects. However, our next aim is to study the presence of these variants in a larger cohort within a different population, since it would bring robustness and confidence to our results. These SNVs could also be tested in older patients with ACS or with other cardiovascular affections.

The on-going CRISPR-Cas9 and sequencing studies may also enlighten us with the functional mechanism that involves these miRNA variants with the onset of ACS and determine if they can become the target of a therapeutic strategy. Our hypothesis is that both variants may have an impact on the levels of the miRNAs in which they have been genotyped. Indeed, rs557283175 is located between miR-199b and another miRNA named miR-3154 and functional assays will help reveal if this variant impacts one or both of these miRNAs. On the other hand, rs117258475 is located within miR-296 mature sequence and thus may have an impact not only in the levels of the miRNA but also in the interaction of its targets. It has already been described that CRISPR-Cas9 can eliminate the expression of a miRNA robustly and efficiently[335,336]. But, to our knowledge, this technology has not been used yet to insert a simple change, as a SNV, in

the sequence of a miRNA gene and in our hands, this strategy is still raising some difficulties.

In summary, this study analyzes for the first time the presence of mutations in miRNA genes related to the onset of ACS in young patients. We found two rare SNVs expressed at a higher frequency in the patients in comparison with the healthy donor population, showing their potential role as genetic prognostic markers of the disease. Both are located within the gene of miRNAs known to be related to cardiovascular diseases, but still further functional studies are guaranteed to understand their role in ACS development and ensure their usefulness as therapeutic targets.

CONCLUSIONS

This project provides novel information on the role of miRNAs and their genetic variants in ACS as biomarkers of the disease development and severity, and even as functional effectors of the disease.

CHAPTER 1 - The data presented show that in young patients, as in the elderly, thromboinflammation is a pathological state underlying ACS. Measurement of NET components in plasma appears to be a useful marker in these patients. We describe here, for the first time, that the miR-SNP rs2431697 of miR-146a could act as an additional risk factor for worse prognosis in ACS patients younger than 45 years. It is proposed that the T allele of rs2431697 may establish a silent proinflammatory state which, upon reaching the appropriate threshold, may predispose to increased NET production and thus promote a higher rate of adverse cardiovascular events.

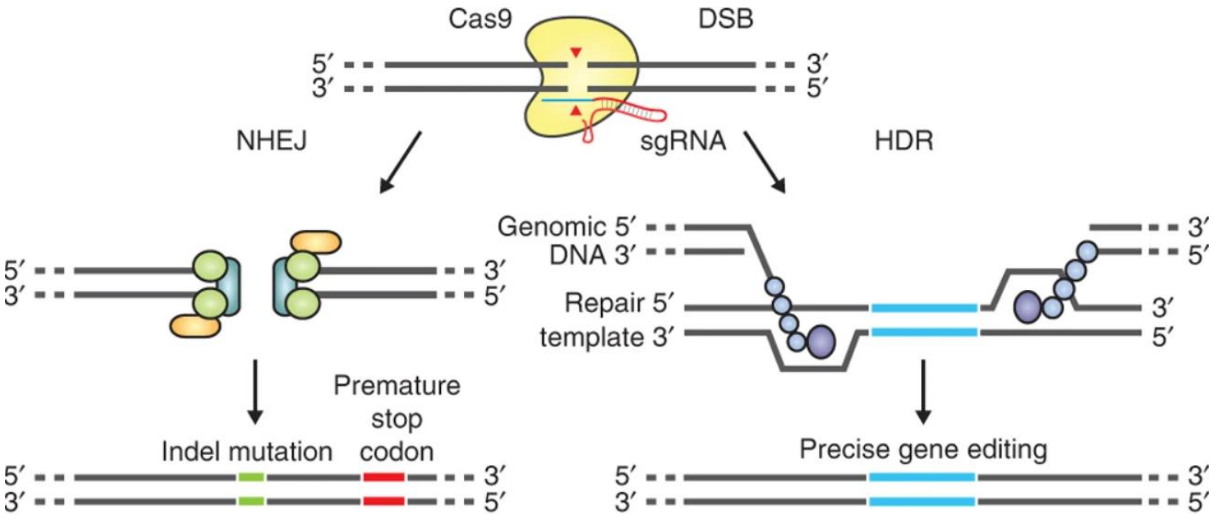
CHAPTER 2 - The present results indicated that histone H4 and, to a lesser extent, DNA, cause an increase in the expression of several coagulation factors. This regulation may alter the hemostatic balance and explain the prothrombotic effect of these NET components. In addition, both DNA and histone H4 triggered an increase in *HNF4A* mRNA expression, which may indicate its partial participation in the regulation of coagulation factors mediated by NET components. In addition, it was determined that histone H4 could induce a decrease in the expression of certain miRNAs of the miR-17/92 cluster, which may explain the upregulation of TF expression induced by histone H4, whereas DNA would act independently of miR. -17/92.

CHAPTER 3 - This study analyzed for the first time the presence of mutations in miRNA genes related to ACS, describing two rare SNVs that are expressed with a significantly higher frequency in the young ACS population compared to the healthy population. The potential role of these two variants in miRNA genes as genetic markers of the disease, their functional implication, and their usefulness as new therapeutic targets in cardiovascular disease, need further evaluation and subsequent confirmation.

APPENDIX

CRISPR-Cas9 functional studies

After identifying potential pathological variants, it is necessary to determine their actual functionality. In recent times, the most recognized and used tool for this aim has been the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology, due to its versatility and its cost-effectivity. In this model, the specificity of the nuclease is based on the complementarity between the guide RNA and the target DNA. The most used system, known as the type II of CRISPR, is normally composed of the Cas9 (CRISPR-associated) endonuclease, responsible for cutting the DNA at the specified location by the guide RNA, noncoding RNA and a unique cluster of direct repeats interspaced by short variable sequences from exogenous DNA targets known as protospacers constituting the CRISPR RNA (crRNA) complex. Each protospacer has to be associated with a protospacer adjacent motif (PAM). When using Cas9, the target can go through two different DNA damage repair pathways: the non-homologous end joining (NHEJ), more susceptible to errors or the homology-directed repair (HDR) which present higher fidelity. The HDR pathway can be used to modify the target locus with precision in the presence of an exogenous repair template that can be conventional double-stranded DNA with homology arms around the desired insertion, or single-stranded DNA oligonucleotides (ssODNs)[337,338] (**App. Figure 1**). When attempting to repress miRNA expression, we have to take special attention to the importance of Drosha and Dicer and select PAMs adjacent to these processing sites[335].



Appendix Figure 1. Schematic representation of the two possible ways of DNA repair used in CRISPR-Cas9. (*Figure from Ran et al., 2013, Nature Protocols*)[339]

CRISPR-Cas9 Assay

For this study, due to the extra difficulty to modify miRNA gene regions, we ought to use several different strategies optimizing the CRISPR assay. Thus, we aimed to desing a CRISPR-Cas9 knock-out (KO) cell model of the whole miRNA gene where the variant of interest is located and a knock-in (KI) model with the specific SNV. For this study, due to the extra difficulty to modify miRNA gene regions, we ought to perform several different strategies optimizing the CRISPR assay:

Strategy 1 - Knock-Out and Knock-n: For both, KO and KI. The simple guide RNA (sgRNA) sequences were chosen using the tool "CRISPR targets" in the UCSC genome browser (University of California Santa Cruz, Genomics Institute) and taking the PAM motif NGG to work with Cas9. It was custom designed by Synthego. This nucleofection of sgRNA (2 different sgRNA for the knock-out and 1 sgRNA for the knock-in) was performed in EA.hy926, as an endothelial model because of the role of endothelium in atherosclerosis development, using a Neon™ transfection System (Invitrogen, ThermoFisher Scientific). Briefly, $5x10^5$ cells were seeded for each condition and replicated 2 days before transfection. On the day of the assay, the cell culture medium was prepared without antibiotics and an assembly of 30 µM sgRNA, 20 µM Cas9 (Alt-R® S.p. HiFi Cas9 Nuclease V3, IDT) and, to a final volume of 7 µL, resuspension buffer R from the Kit Neon Transfection System (Invitrogen, ThermoFisher Scientific) was prepared and incubated for 10 minutes at RT, to create the ribonucleoprotein (RNP) complex. The cells were also resuspended in resuspension buffer R and the Neon Tube of the pipette station was loaded with buffer E from the Kit Neon Transfection System (Invitrogen, ThermoFisher Scientific). 10 µL of the cell suspension was added to each prepared 7 µL of RNP solution. The cell-RNP solution was aspirated with the neon tip where the electroporation takes place. The electroporation conditions are determined depending on the cell type. The cells were then incubated for 2 days.

Strategy 2 - Knock-Out: The second approach was performed in the Genome Engineering & Stem Cell Center (GESC@MGI) from the MacDonnell Genome Institute of the Washington University. Two new sgRNAs were selected and after transfection, the cells were sorted in 6 different medium conditions.

Strategy 3 - Knock-In: To increase the mutation rate, two complementary and overlapping prime editing guides (pegRNAs) were used on opposite sides of the target mutation site. The pegRNAs direct the prime editor protein to the desired locus and encodes the mutation of interest[340].

Strategy 4 - Knock-In: Lastly, a new guide RNA and a ssODN was designed, and RNP nucleofection with Hi-fidelity Cas9 were used to minimize the amount of nuclease re-cutting after donor integration.

The efficacy of the CRISPR transfection was evaluated by PCR and NGS sequencing. Different primers were designed outside and inside the target region and it was amplified by PCR and analyzed by agarose gel electrophoresis. The DNA fragment amplified with the outside primers was purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research), then it was marked with a barcoding sequence to be able to validate multiple samples at once and send to the Center for Genome Sciences & Systems Biology of the Washington University for Illumina sequencing.

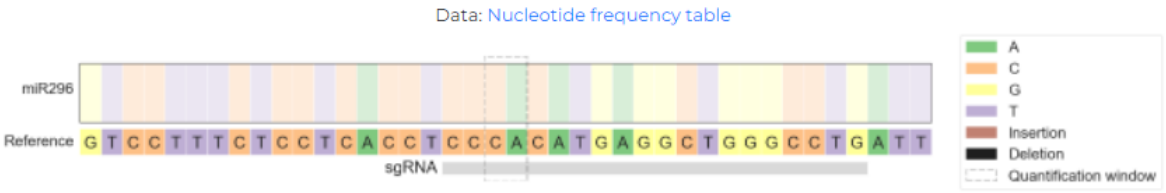
CRISPR-Cas9 functional studies results

So far, due to the time limitation, we had focused on creating a KO model for miR-296 and a KI model of rs117258475, but we plan to create the corresponding model of KI for rs557283175 as well. We had tried 4 different "strategies" to be able to get the desired deletion or mutation. The first approach was also performed in the endothelial cell line, EA.hy926.

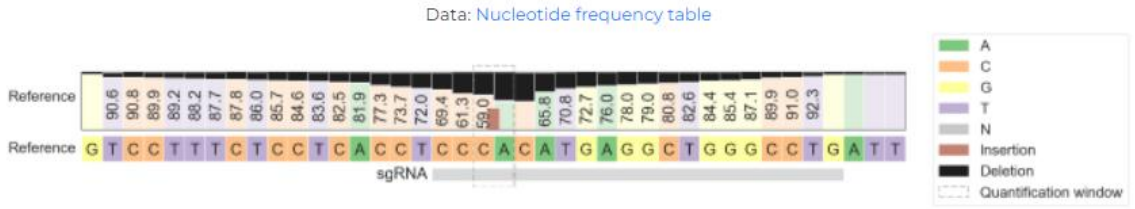
Strategy 1 - KO: After this first approach, we found 92% of modified reads, but when looking at the sequence, there were a high number of different deletions. Therefore we will need a new strategy to avoid these unspecific cuts (**App. Figure 2**).

Strategy 2 - KO: After transfection and cell sorting, we cultured the cells in 6 different medium conditions. In any of the conditions the single cell viability was good enough, the cells took 4 weeks to start growing. Finally, we got only 2 deletion clones ready to collect for further studies. These clones were validated by PCR and agarose gel electrophoresis and by sequencing (**App. Figure 3**).

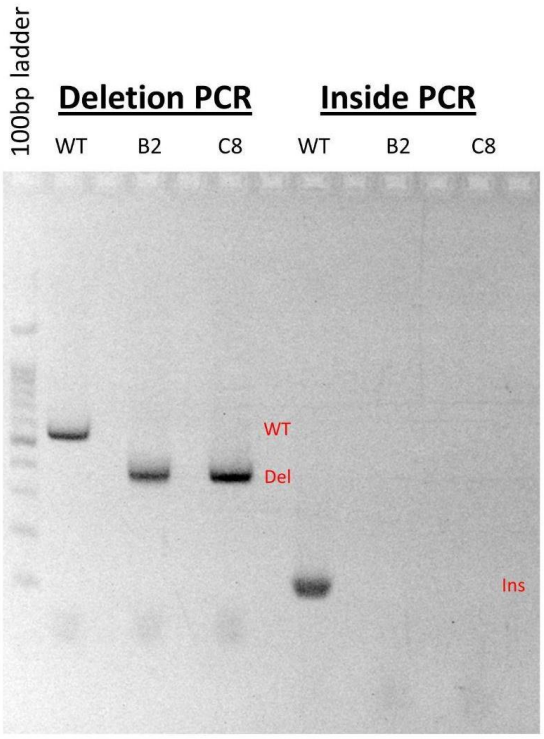
WT



KO



Appendix Figure 2. Nucleotide distribution in WT and the intended miR-296 KO cells after nucleofection of RNP-sgRNA complexes.



Appendix Figure 3. Confirmation of the deletion in the cell clones with a PCR located outside the intended deletion and another PCR inside the intended deletion sequence.

Strategy 3 - KI: Since the microRNA genes make it impossible to introduce a blocking site to prevent re-cut, prime editing technique was used. Unfortunately, the EA.hy926 cells are extremely refractory to plasmid nucleofection and It was impossible to get good survivability after nucleofection with prime editing reagents, therefore this strategy was discarded.

Strategy 4 - KI: After nucleofection with new sgRNAs and ssODN, approximately 3.6% of reads showed the G>A mutation. After sorting the clones, they all showed some degree of extra mutations into the miR-296 sequence. Out of 95 clones identified, none of them had a perfectly clean KI / WT genotype.

At the time of writing this Thesis, the results of this section were still pending. Thus, the NGS sequencing of the mutated clones was on-going to evaluate the changes in mRNA expression in those cells carrying the SNP.

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SCIENTIFIC PRODUCTION

FIRST AUTHOR ARTICLES INCLUDED IN THIS THESIS

1. **de los Reyes-García AM**, Rivera-Caravaca JM, Zapata-Martínez L, Águila S, Véliz-Martínez A, García-Barberá N, et al. **MiR-146a Contributes to Thromboinflammation and Recurrence in Young Patients with Acute Myocardial Infarction.** J Pers Med. 2022 ;12(7):1185.

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1. **de los Reyes-García AM**, Arroyo AB, Teruel-Montoya R, Vicente V, Lozano ML, González-Conejero R, et al. **MicroRNAs as potential regulators of platelet function and bleeding diatheses**. Platelets. 2019 ;30(7):803-8.

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