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"Fibrillazione atriale nell'anziano: dalla valutazione clinica alle indagini di metabolomica.
Un approccio 'multidimensionale' per lo studio di un'aritmia complessa."

*"Atrial fibrillation in the elderly: From multidimensional assessment to a metabolomic insight.
A complex approach for a complex arrhythmia."*

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Abstract

Atrial fibrillation (AF) is the most common type of arrhythmia among the elderly and it is characterized by a disordered electrical activity of the atria which causes ineffective atrial contraction. The major riskes posed by AF are the occurrence of stroke, worsening heart failure, and dementia. Its pathophysiology is complex and multifaceted and several aspects remain not completely understood. Our project aimed at exploring the molecular characteristics of AF patients with an untargeted metabolomics approach followed by a more in-depth analysis focused on the lipidomics profile, acylcarnitine and amino acids concentrations. In parallel, patients' frailty was evaluated with the tools from the Geriatric Multidimensional Assessment and the CHA_2DS_2-VASc score, and key markers of inflammation including IL-6 and OPG were measured to assess the link between low-grade inflammation and AF development and progression. All these data were analyzed and integrated to have a comprehensive understanding of the interplay between all the factors included in the study. A difference in BMI between patients and healthy controls emerged and this may represent a surrogate marker of sarcopenia. With the cluster analysis applied to metabolomics data, a cluster with higher IL-6 levels, higher CHA_2DS_2-VASc score, and lower physical function was detected, meaning that metabolomics could group patients according to their overall clinical profile and the subjects with the arrhythmia presenting the worse metabolic profile could represent the frailest ones. An association between IL-6 and medium- long-chain acylcarnitines emerged from the analysis shedding light on the complex interplay between low-grade inflammation and acylcarnitines which can alter the heart electrophysiology and thus contribute to the establishment of a favourable substrate for AF development. Additionally, a decrease in arginine levels according to age and progression of disease was found and this could represent a marker of endothelial dysfunction. All these findings, linking bench with bedside experience, could be useful to guide the clinical management of patients with AF according to age. In particular, these data may help in the choice between rate and rhythm control therapy of the arrhythmia and could be the basis to understand the correlation between AF and frailty.

1 INTRODUCTION

1.1 Sinus rhythm and the cardiac conduction system

The cardiac conduction system (CCS) is composed by a series of specialized tissues which are responsible for the initiation and the coordination of the heartbeat. The main components are the sinoatrial node (SAN), the atrioventricular node (AVN) and the bundle of His with the associated Purkinje fibers (Fig. 1.1).

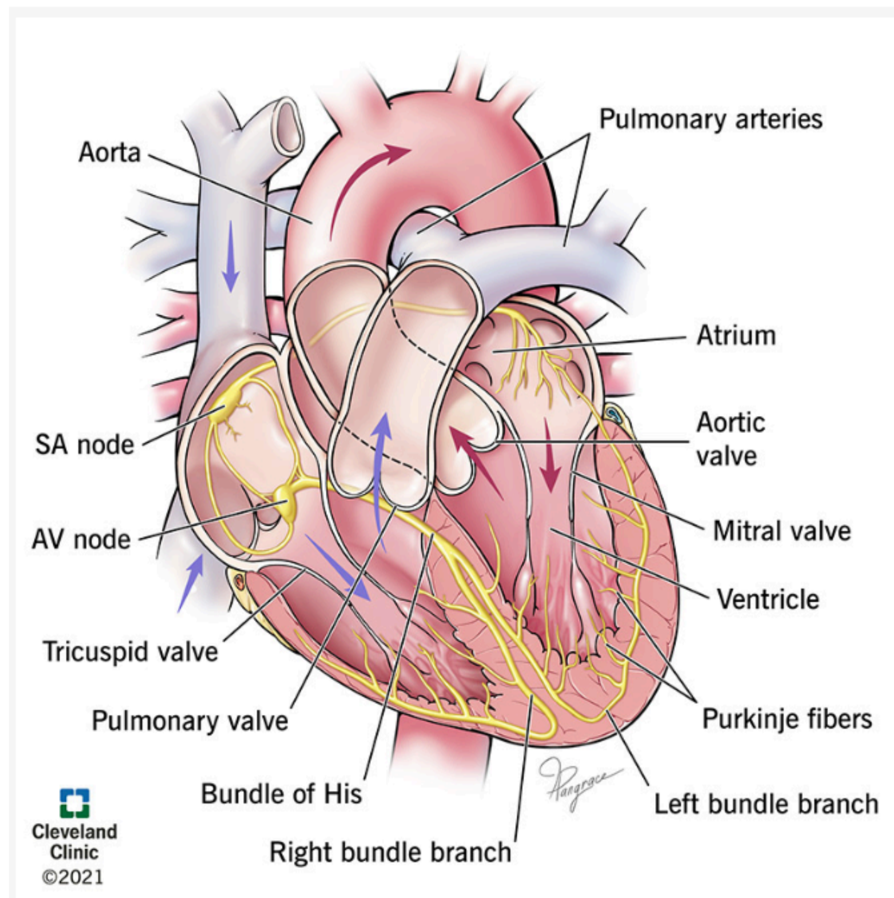


Figure 1.1: Anatomy of the heart and its cardiac conduction system.

The SAN, situated in the upper part of the right atrium, is the heart pacemaker and is responsible for the initiation of the cardiac action potential. Once started, the action potential propagates to the rest of the heart generating the heartbeat. Atria and ventricles are separated by a fibrous tissue ring and thus the action potential can only

pass through the AVN located at the base of the right atrium. The conduction of the action potential through the AVN is slow as a delay between the atrial and the ventricular contractions has to be introduced to allow the atria to first pump blood in the ventricles, which then contract to deliver blood to the body. Additionally, the AVN works as a back-up pacemaker in case of SAN malfunctioning and in cases when the atria beat too rapidly, as it occurs in atrial fibrillation (see Section 1.2.1), the AVN limits the quantity of action potentials reaching the ventricles[1]. Another main component of the CCS is the bundle of His located in the ventricles. It is divided into two branches, one in the right and one in the left side of the heart, each ending in a network of Purkinje fibers. This system spreads the action potential rapidly to both ventricles to ensure their simultaneous contraction. A dysfunction in even just one of the components of the CCS may lead to irregular heartbeats and interruptions of the propagation of the action potential. When the system works properly, the regular, physiological heartbeat is called sinus rhythm and is defined by the PQRST complex as shown in the electrocardiogram (ECG) trace in Fig. 1.2. Each letter or group of letters indicates a specific phase of the heartbeat. The P wave is produced by the SAN and indicates atrial depolarization; the QRS wave is produced by the action potential after having crossed the AVN, representing ventricular depolarization. This is followed by the T wave which corresponds to the ventricular repolarization phase.

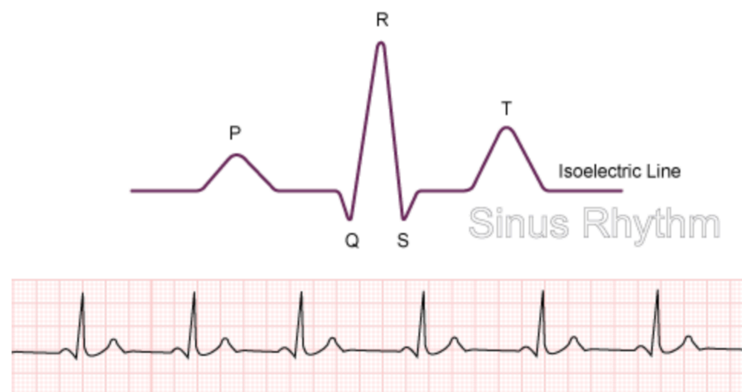


Figure 1.2: Above: the heartbeat phases defined by the letters PQRST. Below: an ECG trace showing regular sinus rhythm.

1.2 Atrial fibrillation

1.2.1 Definition and classification

Atrial fibrillation (AF) is a supraventricular tachyarrhythmia characterized by an uncoordinated atrial electrical activation with a consequent ineffective atrial contraction. AF differs in its presentation, duration, and spontaneous termination, and a classification based on these factors is adopted[2] (Fig. 1.3):

- First diagnosed: AF that was not diagnosed before, regardless of its duration or the presence and severity of symptoms;
- Paroxysmal: AF terminating spontaneously or with a medical intervention within 7 days;
- Persistent: AF that continues beyond 7 days, including the episodes terminated by pharmacological or electrical cardioversion after more than 7 days;
- Long-standing persistent: AF lasting more than 12 months, for which a decition to follow a rhythm control strategy was then adopted;
- Permanent: AF that is accepted by the patient and the physician and for which no further attempts to restore or maintain the sinus rhythm will be undertaken. Permanent AF is thus a decision of the patients and the physicians, often motivated by a pathophysiology hindering the possibility to restore a stable sinus rhythm. If a rhythm control strategy is successfully adopted, then the arrhythmia will be re-classified as long-standing persistent AF.

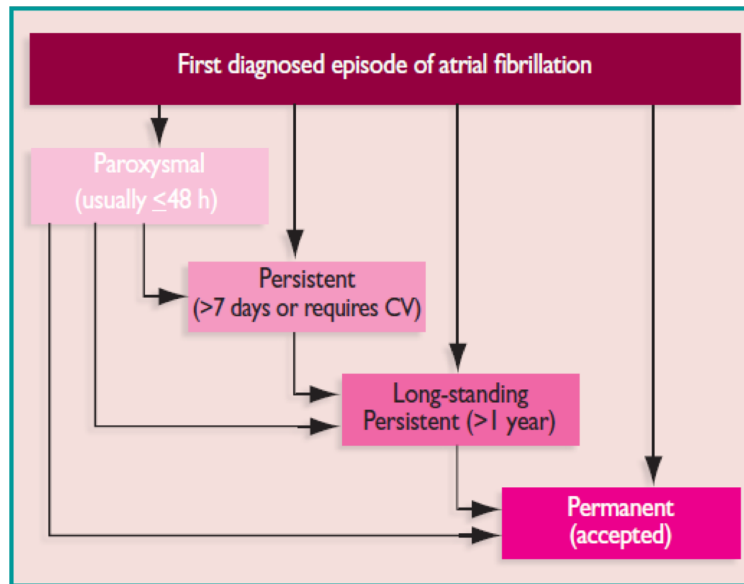


Figure 1.3: Classification of AF types. From Camm et al. 2010.

AF is a multifaceted arrhythmia that presents variations in outcomes and can have an impact on several aspects of the life of patients. For these reasons, the study of AF requires an integrated, multidisciplinary approach.

1.2.2 Pathophysiology

Research efforts are being directed towards gaining information about AF pathophysiology to both understand the molecular mechanisms that lead to the onset of the arrhythmia and to analyze its natural course in patients in order to highlight the most effective treatments. AF, in fact, is the result of a complex interplay of triggers, perpetuators, and the development of a substrate that together favour the occurence of the arrhythmia. A favourable susbtrate is usually characterized by a dilation of the left atrium and the presence of fibrosis, with a consequent delay in the electromechanical conduction[2]. Several other factors may cause alterations of atrial function, such as myocardium hypercontractility, inflammation, vascular remodelling, ischemia, ionic channels dysfunctions, and instability of calcium levels. All these factors can contribute to the formation of ectopic circuits that may lead to a dysruption of the heart impulse conduction, thus increasing the propensity to the development of AF and facilitating the

hypercoagulable state associated with AF[2].

1.2.3 Epidemiology

AF is the most frequently sustained arrhythmia in the adult population worldwide. It is known to especially affect the elderly population and, in 2016, its prevalence was set to about 45 million people worldwide[3]. It is estimated that 2 to 4 % of the world adult population is affected by AF, and some models demonstrated that this number is destined to increase in the next years, due to the aging process of the population and the improvement in overall diagnostic ability[4–6]. The risk of developing AF throughout one's life depends on several genetic and subclinical factors. However, the main risk factor for the onset of AF remains advanced age. Common comorbidities are hypertension[7], diabetes mellitus[8], heart failure, coronary artery disease, chronic kidney disease[9], and obsructive sleep apnea[10] (for further details, see Section 1.2.4). Other risk factors, such as diet and lifestyle, can be modified and a control over obesity and alcohol or stimulants consumption helps reducing the risk of developing AF[11]. Therefore, changes in lifestyle may prevent the onset of AF, slow its progression, and reduce hospitalization and mortality due to cardiovascular diseases[12].

1.2.4 Clinical aspects and complications

AF represents one of the major causes of morbidity and mortality associated with cardiovascular diseases and the presence of AF increases the incidence of stroke, heart failure, dementia, and hospitalizations. It is estimated that, overall, AF increases the risk of stroke and systemic embolism about five times, according to the presence or absence of other specific risk factors[2,13]. A tool to evaluate the probability of stroke onset for patients is the CHA_2DS_2-VASc score[14]. The CHA_2DS_2-VASc is a clinical score widely used to evaluate thromboembolic risk in patients with AF[15] and oral anticoagulant therapy recommendations are given based on this score[16]. This scoring system gives a score from 0 to 9 according to the presence of certain risk factors that determine the probabilty of the patients to experience a stroke. In particular:

- **C**: Congestive heart failure - 1 point, if the patient presents heart failure or evidence of left ventricular dysfunction or hypertrophic cardiomyopathy;
- **H**: Hypertension - 1 point, if the patient has hypertension or is following a therapy for hypertension;
- **A**: Age 75 years or older - 2 points, if the patient is 75 years or older;
- **D**: Diabetes mellitus - 1 point, if the patient has diabetes, takes hypoglycemic drugs, and/or insulin, or if has fasting glucose levels > 125 mg/dL (7 mmol/L);
- **S**: Stroke - 2 points, if the patient had previously experienced stroke, transitory ischemic attacks or thromboembolic episodes;
- **V**: Vascular disease - 1 point, if the patient has coronary artery disease that is relevant to angiography, had a myocardial infarction peripheral artery disease or atherosclerosis;
- **A**: Age 65 - 74 years - 1 points, for patients aged between 65 and 74;
- **Sc**: Sex category - 1 point, if the patient is a woman, only if another risk factor is present.

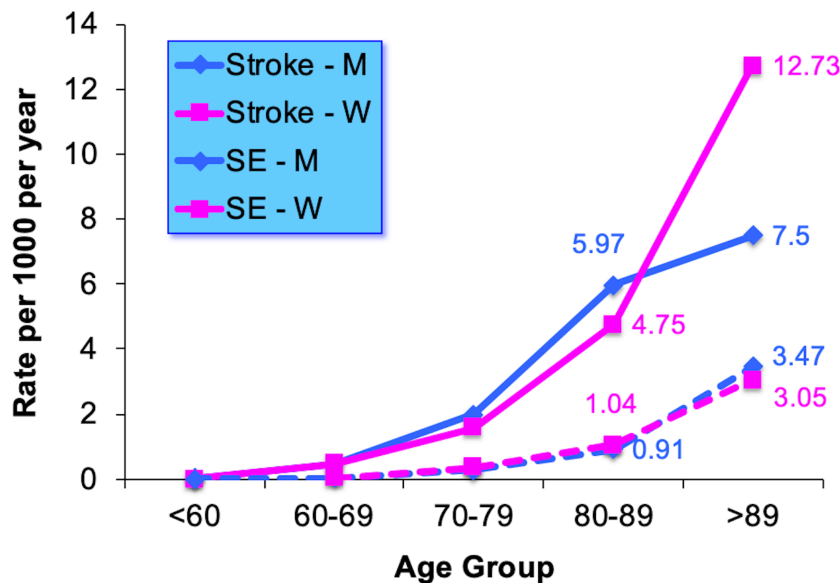


Figure 1.4: Age-specific rates of first ever AF-related incident ischaemic stroke and systemic embolism (SE) in the Oxford Vascular Study (2002-2012; N = 92728; 9 general practices - about 100 family doctors)

Beside being a major cause of morbility and mortality for cardiovascular causes, AF also affects the overall patients' quality of life (QoL). In fact, studies show that AF patients have a reduced QoL compared to healthy subjects and their level of QoL is comparable to that of patients with coronaropathies or those who experienced myocardial infarction[17]. Furthermore, AF is demonstrated to have psychological effects on patients which report an increase in anxiety and depression[18]. AF association with the probabilty of experiencing cognitive decline and the onset of dementia is well known[19,20]. Although AF and dementia share some common risk factors and are both associated with ageing, AF seems to be independently associated with different forms of dementia, including Alzheimer's disease[20]. This link between AF and congnitive decline may be a manifestation of micro and macro clots occuring as a consequence of the cardiac dysfunction[21]. AF often comes with several comorbidities, some of which also affect the cardiovascular system (Fig. 1.5). The most frequently encountered are:

- **Hypertensive cardiopathy**. At least 40 % of patients with AF present arterial hypertension[22]. Hypertensive cardiopathy is characterized by left ventricular hypertrophy caused by a pressure overload due to hypertension[23], and diastolic dysfunction in patients with arterial hypertension. The identification of this condition is crucial, because these types of patients are more prone to develop heart failure, arrhythmias, myocardial infarction, and sudden death[24].
- **Coronary artery disease**. Myocardial infarction increases the risk of developing AF by 60-70 %[25]. The coexistence of AF and acute coronary syndrome (ACS) is correlated with a worse prognosis and a suboptimal anticoagulant treatment compared to patients with ACS but no AF[26].
- **Valvular heart disease**. About 33 % of patients with AF have a form of valvulopathy and almost any valvular lesion that leads to a significant level of stenosis or valvular regurgitation is associated with the development of AF[25].
- **Heart failure (HF)**. Beside sharing many risk factors such as hypertension, diabetes, and valvular heart disease, AF and HF are intertwined so that HF begets AF and *vice versa*. When they are both present, patients' prognosis is worse[27]. HF subtypes are classified according to the left ventricular ejection fraction (LVEF):

HF with reduced EF (HFrEF) occurs when LVEF is $\leq$ 40-45 %; HF with preserved EF (HFpEF) presents LVEF $\geq$ 45-50 %; a third category has been established by the *European Society of Cardiology* (ESC) for the "gray zone" with 41 % $\leq$ LVEF $\leq$ 49 % (HFmrEF, heart failure with mid-range ejection fraction). HF subtypes affect left atrial (LA) remodelling differently: HFrEF causes greater eccentric LA remodelling than HFpEF, but the latter increases LA stiffness[28]. Both modifications contribute to the setting of a favourable substrate for AF development. A study showed that more than half of the subjects with HF had AF at some point and AF was more likely to precede HF rather then developing afterwards; more than one third of patients with AF had HF which mostly developed after AF[29].

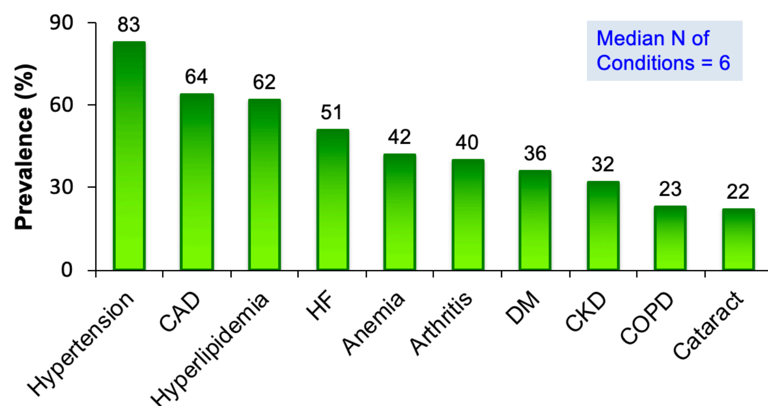


Figure 1.5: Ten most common chronic comorbid conditions among Medicare beneficiaries with AF. Beneficiaries > 65 y of age; N = 2.426.865. From Rich et al. 2016.

Other comorbidities are not cardiovascular diseases *per se*, but are linked to AF in different ways. The most frequently observed in AF patients are the following:

- **Obstructive sleep apnea** (OSAS, Obstructive Sleep Apnea Syndrome). While OSAS is a common condition, it remains frequently undiagnosed. It is characterized by a partial or total obstruction of airways that may cause a physiological disorder characterized by at least five episodes of apnea or hypopnea for every hour of sleep. The prevalence of AF among patients with OSAS is 2-4 fold higher than in subjects without breathing sleep disorders[25]. According to the

Sleep Heart Health Study carried out in 2006, OSAS is more prevalent in patients with AF compared to the general population[30]. AF and OSAS have several risk factors in common, such as hypertension, obesity, and diabetes. Additionally, OSAS favours inflammation, as demonstrated by higher levels of C-reactive protein, interleukin-6, and tumor necrosis factor alpha (TNF-α) in serum of patients with this breathing disorder; OSAS also leads to hemodynamic changes that contribute to the enlargement of the atrial chamber, atrial fibrosis, and pulmonary vases remodelling[31]. An adequate treatment of OSAS helps patients with AF to achieve a better prognosis[25].

- **Chronic Obstructive Pulmonary Disease** (COPD). Patients with COPD have a higher incidence of AF and COPD is present in 10-15 % of patients with AF[32]. COPD is one of the predictors of progression of AF from paroxysmal to persistent forms[33]. The onset of AF in patients with COPD may significantly worsen the symptoms at pulmonary level because of the irregular heartbeat and the reduced filling of ventricles, which lead to a worse prognosis for the patient. AF management in COPD patients is complex because drugs used to improve pulmonary function may cause tachyarrhythmias and drugs to manage AF may cause bronchospasm[33].
- **Chronic kidney disease** (CKD). The incidence of CKD is correlated to AF and, when they coexist, the patient's prognosis is worsened. The possible mechanisms by which CKD may affect AF onset involve the triggering of a pro-inflammatory state and the activation of the renin-angiotensin-aldosterone system that lead to atrial fibrosis as well as electrical and structural remodelling that contribute to the development of a favourable substrate for AF[25]. However, the relationship between AF and CKD is bidirectional, meaning that AF also triggers CKD and patients with AF have an increased risk of developing CKD, which is present in about 15 % of AF patients[34].
- **Inflammation**. A chronic low-grade proinflammatory state is often developed with age and this represents a risk factor for several co-existing pathologies (multimorbidity), physical and cognitive disability, frailty, and even death[35,36]. Given the prominent role that inflammation seem to play in the development of age-related

diseases, targeting inflammation may be a crucial step to promote healthy, successful ageing[37], which can be accomplished if the molecular, cellular, and physiological mechanisms that contribute to the functional changes associated with aging are well understood.

1.2.5 Diagnosis

Detecting AF is not always an easy task since silent AF episodes are fairly common. The incidence of asymptomatic AF depends on the frequence of monitoring and the burden of AF in the cohort of patients being examined[38]. As an example, a study reported the presence of silent supraventricular arrhythmias in 58 % of patients that were being monitored for other heart conditions[39], while another found 39.7 % of patients enrolled in the study to be asymptomatic[40]. Thus, when silent, AF can only be accidentally diagnosed during heart screenings performed for other purposes. However, the majority of AF patients experience symptoms which include palpitations, fatigue, and dyspnea which can negatively affect the patient's everyday life. Evaluating symptoms is important for both choosing the most suitable therapy and for the diagnosis of AF itself, as certain symptoms may reveal some underlying cardiovascular risk factors or undiagnosed pathological conditions. For symptom evaluation, the scale proposed by the *European Heart Rhythm Association* is widely used; with this method, the presence and impact of symptoms is evaluated using a scale from 1 to 5[41]. AF is defined "clinical" when it presents symptoms or if it has been diagnosed with an electrocardiogram (ECG). In order to diagnose a clinical AF, it is necessary to perform an ECG with one lead indicating the presence of AF for at least 30 s or a 12-lead ECG[2,42]. When the atrio-ventricular conduction is not compromised, the typical ECG profile of AF is characterized by irregular R-R intervals, lack of distinct repeated P waves, and irregular atrial activations (Fig. 1.6).

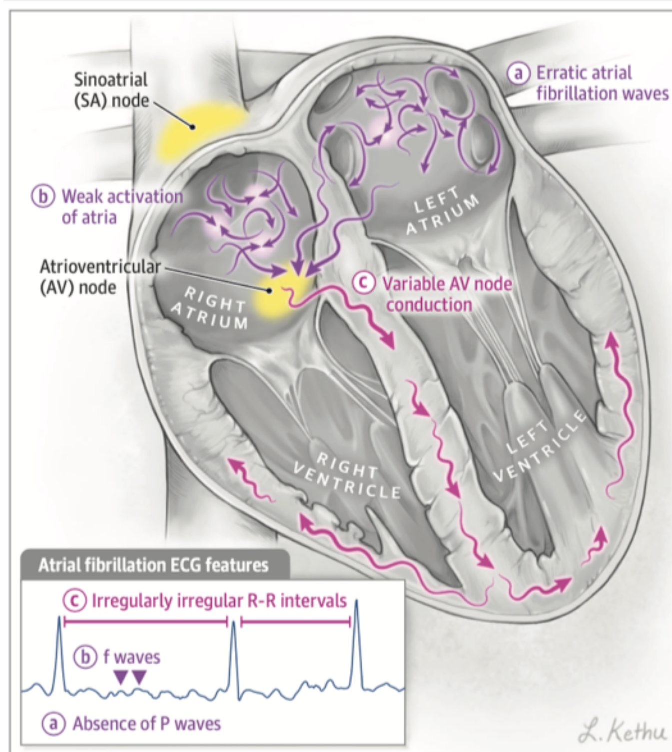


Figure 1.6: Dynamics of atrial fibrillation.

1.2.6 Therapy and management

When dealing with an AF patient, a physician has to evaluate which route of intervention is the most suitable. In particular, one important choice is to whether attempt to restore the sinus rhythm or to focus on controlling the heart rate while leaving the heart rhythm as it is. These two strategies are called rhythm control and rate control, respectively. They both present advantages and disadvantages according to the clinical case and both are supported by anticoagulant therapy.

- Rhythm control strategy: the sinus rhythm can be restored by electrical cardioversion (ECV) or pharmacological cardioversion (PCV) with antiarrhythmic drugs (see Section 1.2.7). The rationale behind this choice is the possibility to alleviate or eliminate the symptoms and to increase the patient's tolerance toward physical activity. Beside a better QoL, restoration of sinus rhythm also

reduces the risk of stroke and increases the probability of survival, in case the sinus rhythm is maintained over time[43].

- Rate control strategy: this approach, often secondary, is usually adopted only if the rhythm control strategy fails[44]. It represents an alternative that allows for the control of the rate of the ventricular response to AF through the employment of drugs that block the atrioventricular node or by ablation of the atrioventricular junction followed by the installation of a pacemaker[45]. This approach makes the therapy simple and allows for the use of drugs that are less impacting than antiarrhythmic drugs[43].

Another important decision the physician has to make is to evaluate if the patient needs oral anticoagulant therapy to prevent stroke or emboly. Two kinds of anticoagulants can be prescribed:

- Vitamin K antagonists (VKA). An appropriate dosage of VKA reduces the risk of stroke and systemic embolism by 64% and all-cause mortality by 26%[7]. VKA are currently the only safe treatment for patients with mitral valve stenosis and/or an artificial valve[2]. However, their use is limited by a quite narrow therapeutic window that requires frequent monitoring of the INR (International Normalized Ratio) and consequent adjustments of the dose[46]. An example of this type of drug is warfarin.

- Novel oral anticolagulants (NOAC). A meta-analysis on NOAC showed a favourable risk-benefit ratio with a significant decrease in stroke, intracerebral hemorrhage, mortality, and bleeding levels comparable to warfarin except for the gastrointestinal region where they appear to be more frequent[47]. Compared to VKA, NOAC are more simple to use and the therapy is easier to follow thank to their better pharmacokinetic profile[48] and for their safety and efficacy, especially in more vulnerable patients such as the elderly, those with kidney dysfunctions or those that experienced a stroke[49].

The approach to AF requires an integrated and coordinated management of the patient, where therapeutic options are discussed by an interdisciplinary team and personalized according to the patient's needs. The treatment of choice can then be modified in time according to the evolution of AF, the onset of new risk factors or symptoms, and the rise of new therapeutic strategies[2]. The involvment of the patients, their family and caregivers is essential for an effective management of the pathology[12].

Recently, a model called *Atrial fibrillation Better Care (ABC) pathway* has been proposed[19]. This approach is based on three main goals:

- A: *Avoid stroke*. The prevention of stroke represents an absolute priority in the management of AF. Strokes caused by the arrhythmic substrate of AF more frequently have a fatal or imparing outcome compared to strokes with different etiology[7].

- B: *Better symptom management*. According to the symptoms, patient and physician define the best strategy to reduce the impact of symptoms in the everyday life of the patient, thus improving his QoL.

- C: *Cardiovascular and other comorbidities*. Control other pathologies, cardiovascular and non-cardiovascular, that coexist with AF. In fact, part of the integrated care of the patient consists in controlling risk factors and comorbidities, like blood pressure, heart failure, diabetes, nocturnal apneas or cardiac ischemias, with the goal of reducing the probability of stroke and cardiovascular burden[12].

A multidisciplinary approach which includes risk factors and lifestyle management is crucial to prevent the onset of AF in patients with predisposing factors (Fig. 1.5). Modifiable risk factors that can be controlled include:

- Hypertension. Studies show that long-term blood pressure control lowers the incidence of new-onset AF, while an inadequate control of blood pressure in patients $\geq$ 65 years is linked with an increased risk of new-onset AF[23]. Thus, current

guidelines recommend an appropriate control of blood pressure as a primary prevention tool against AF[50].

- Obesity. Individuals with a BMI > 30 kg/m^2 have a higher risk of developing AF and a study from the Framingham Heart Study highlighted that every unit increase in BMI is associated with an increased risk by 4-5 %[51]. In case of subjects already diagnosed with AF, it is demonstrated that obesity favours the progress of the arrhythmia from paroxysmal to permanent[52]. This link between obesity and AF can be explained as pericardial and epicardial fat contributes to build an arrhythmogenic substrate by affecting ionic currents and thus reducing cellular action potential[53]. For these reasons, long-term weight loss is associated with a reduction of AF burden and a possible reversion of the type and progression of AF[54].
- Diabetes. The risk of developing AF is 3 % higher for each year of having diabetes, and this risk was demonstrated to negatively affect glycaemic control[55]. For people with diabetes mellitus it is advised to screen for AF, as autonomic dysfunction may favour silent AF episodes[56].
- Alcohol. Chronic, heavy alcohol consumption results in a consistent linear increase in the risk of AF[57]. Alcohol can trigger arrhythmias as it has a direct toxic effect on cardiomyocites, but also involves mechanisms that may affect the autonomic nervous system, cause metabolic electrolyte imbalances (like hypokalemia and acidosis), and alter atrial electrical properties[58].
- Physical activity. Intense sport activity increases the risk of AF, mostly due to an enlargement of the left atrial diameter[59]. However, moderate physical activity helps to prevent AF and, in particular, multicomponent training including aerobic, muscle strenghtening, balance, stretching and coordination exercises seem to give the most benefit[25]. Subjects already diagnosed with AF also benefit from this type of exercise that can have an impact in the overall QoL. A study demonstrated that 1 h of yoga twice a week helped improving patients' symptoms, heart rate, blood pressure, overall arrhythmia burden, as well as anxiety and depression scores[60].
- Smoke. Cigarette years appear to be directly proportional to an increased risk of incident AF, with current smokers having a higher risk than former smokers

with similar cigarette years[61]. Smoking also predisposes to AF as it is linked with the development of conditions contributing to AF development, such as COPD, myocardial infarction, and HF.

1.2.7 Cardioversion

Cardioversion, i.e. the restoration of sinus rhythm, can be achieved in two ways: pharmacological cardioversion (PCV) with antiarrhythmic drugs (e.g. amiodarone), and electrical cardioversion (ECV). ECV is performed under general anesthesia and a cardioverter/defibrillator is used. Electric shocks are delivered through adhesive pads placed in anteroposterior position. Several studies show that the efficacy of external, monophasic direct-current cardioversion in patients is 80-85 %[62]. The efficacy is even higher when using biphasic defibrillators, which apply both positive and negative currents, require fewer shocks and less energy to deliver, and are less prone to cause dermal injury[63]. An example of biphasic wave is shown in Fig. 1.7.

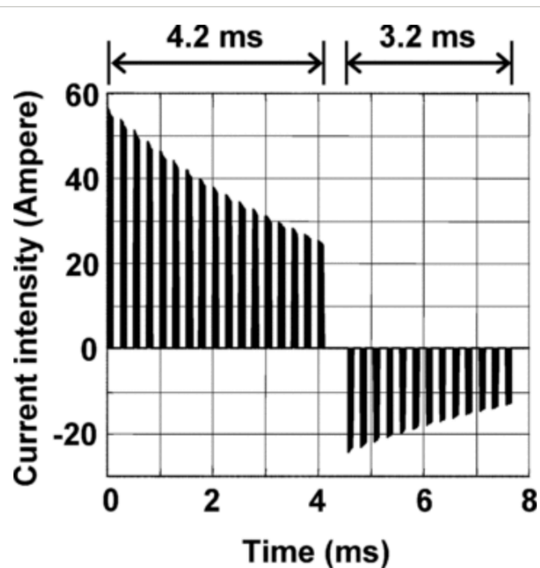


Figure 1.7: An example of a multipulse biphasic wave used for ECV. Adapted from Fumagalli et al. 2009.

The benefits of the restoration of sinus rhythm not only involve the heart itself but have a wider impact. In fact, with AF, several haemodynamic imbalances may develop, in-

cluding the loss of atrial contractility and the atrio-ventricular coordination that affect the ventricular filling and thus the cardiac output[20]. These hemodynamic imbalances have an impact on the brain and studies show that the ECV with sinus rhythm restoration improves brain perfusion and consequently reduces the risk of cognitive decline[64].

1.3 Frailty and the Geriatric Multidimensional Assessment

Aging is frequently characterized by the coexistence of several comorbid conditions, often reciprocally interacting to produce a negative impact on health status. These changes, together with sub-clinical malnutrition and low-grade inflammation, are associated with frailty development, a phenomenon tipically related to aging, which is characterized by an increase in vulnerability to stressors and a decreased ability to maintain homeostasis[65]. Cardiovascular health is also importantly affected by the process of aging, thus the management of cardiovascular diseases in the elderly represents a challenge. In fact, AF onset in older subjects could be linked to the fast progression of disability and frailty and the arrhythmia is considered by physicians as a marker of a frail condition[66]. In AF patients, the CHA_2DS_2-VASc score can also be considered as an indicator of frailty. In fact, it has been found that the CHA_2DS_2-VASc score correlates with cognitive status, depressive symptoms, and physical performance of patients with AF measured through the tools of the Geriatric Multidimensional Assessment (GMA)[67]. A geriatric comprehensive assessment is essential to evaluate frail elderly people in a medical setting. In fact, delineating a frail profile plays an important role in the identification of high risk patients, their clinical management, and prognosis. For this reason, researchers identified different multidimensional instruments to identify frailty considering the complexity of the geriatric patient, specifically focusing on physical function, cognitive ability, and general mood[68]. The questionnaires that are mostly used are:

- Short Physical Performance Battery (SPPB). This test is a well-established tool to assess lower extremity physical performance status and to evaluate the functional capability and frailty condition of elderly individuals[69]. The SPPB is based on three timed tasks: standing balance (ability to stand with feet side by

side, in semi-tandem, and in tandem position), walking speed (time needed to walk a 4-meter distance), and chair stand tests (time employed to stand 5 times from a chair and return to the sitting position). The timed results from each task are summed to obtain a score ranging from 0 to 12, with a score < 6 identifying subjects at higher risk of disability and mortality[70,71].

- Mini Mental State Examination (MMSE), an 11-item questionnaire that is used to assess the cognitive status of patients. It explores subject's memory, orientation, attention, comprehension, language and calculating skills, and the ability of performing more elaborate tasks, such as copying a complex drawing[72]. Scores range from 1 to 30; a score < 24 is indicative of cognitive decline[73].

- Geriatric Depression Scale - Short Form (GDS-SF), a 15-item screening and diagnostic tool to detect depressive symptoms in older subjects[74]. To each query, patients can answer "yes" or "no" and a score > 5 suggests the presence of depression[75].

1.4 Metabolomics

Metabolomics is the field of life science that aims at characterizing metabolites from cells, organs, tissues, or biofluids using advanced analytical chemistry techniques[76]. A metabolite is defined as a small molecule with a molecular mass $< 1{,}500$ Da that can be detected in a specific cell, organ, or organism[77]. Metabolites can be the products of endogenous catabolism or anabolism, such as lipids, sugars, amino acids, short peptides, nucleic acids, alcohols, or organic acids. These are called primary metabolites because they are encoded by the host genome and are essential for the development and physiological functioning of the organism[78]. Certain primary metabolites, like some essential amino acids and vitamins, are not produced by the organism and therefore need to be acquired from the diet. Molecules that are not necessary for the organism, but are still incorporated in the metabolism, are the secondary metabolites which include, among others, food additives, drugs, pollutants, pesticides, and micro-

bial byproducts. This collection of exogenous metabolites is also defined "exposome" and it encompasses, at its most complete, the exposure to the environment throughout the lifetime of an individual[79]. Researches in metabolomics have been highlighting the role that small molecule metabolites play in many biological processes. In fact, they are not merely the products of metabolism, but often have roles as signalling molecules, immune modulators, or environmental sensors[78]. The metabolome varies according to internal and external factors such as age, diet, circadian rhythm, environment, geographical location, gender, and one's own genetics[80,81]. Being so sensitive to stimuli and signals, metabolites are considered "the canaries of the genome" because a single DNA base change in a gene may lead to a 10,000-fold change in the levels of endogenous metabolites[82]. This amplification effect is due to the fact that metabolites are also the downstream products of genes, transcriptional activators, RNA transcripts, protein transporters, as well as enzymes[83]. This potential to "read" what is happening in the organism is one of the reasons why metabolomics is increasingly being applied in many types of exploratory physiological studies and, particularly, in biomedical research. Considering that a microbe, such *Escherichia coli*, has more than 3,700 small molecules and that the metabolome of a yeast like *Saccharomyces cerevisiae* counts about 16,000 metabolites, it is believed that the human metabolome probably consists of more than a million compounds[84,85]. These compounds belong to several different chemical classes and this adds a layer of complexity to the possibility of finding a unique method to measure all of them. This is why metabolomics is approached with different methods and instruments depending on the research question to be tackled and, in case of *targeted* metabolomics, on the molecules of interest. Analytical tools generally used in metabolomics include nuclear magnetic resonance (NMR) spectrometers, mass spectrometers (MS), gas chromatography (GC), liquid chromatography (LC), ion mobility (IMS), and capillary electrophoresis (CE) systems. These tools can all be coupled with MS, so we can have gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), ion mobility spectrometry-mass spectrometry (IMS-MS), or LC-MS/NMR.

Mass spectrometry-based metabolomics provides qualitative and quantitative analyses

with high selectivity and sensitvity, as well as the potential to identify metabolites. When combined with a separation technique, metabolites are also separated in a time dimension, thus providing additional information on the molecules and their physico-chemical properties. GC-MS has been labeled as the *gold standard* in metabolomics, meaning that each new approach should be compared against this method with respect to breadth, sensitivity, and specificity of metabolite detection[86]. This definition is due to the several advantages that this technique presents. The combination of GC with electron ionization MS (EI-MS) provides high chromatographic resolution, analyte-specific detection and quantification of metabolites[87]. It also allows the identification of unknowns as the rich and complex fragmentation pattern generated by EI helps increasing the accuracy of mass spectral matching. Data about mass spectra and retention times obtained under standardized conditions (70 eV electron ionization energy) have been collected over the years in dedicated libraries, such as the NIST Mass Spectral Library collection of the U.S. National Institute of Standards and Technology[88], the Wiley registry[89], the MassBank database[90], and the Golm repository[91]. In order to be processed in GC-MS a molecule needs to be volatile and thermally stable. Most metabolites have high boiling points and therefore a derivatization step is required. The derivatizion method mostly used in metabolomics analyses by GC-MS is trimethylsilylation[92,93] which removes acidic protons from hydroxyl, carboxyl, amino, or thiol groups (Fig. 1.8). One disadvantage of this derivatization technique is that silylation is highly sensitive to moisture. This is why the sample extract must be thoroughly dried before derivatization in order to avoid hindering of the efficiency of the reaction or derivatives degradation[87].

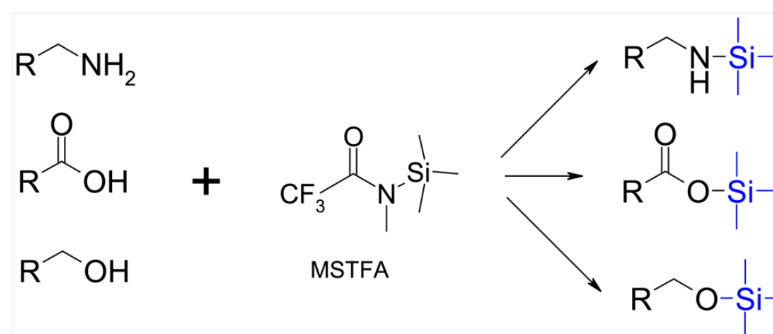


Figure 1.8: Silylation mechanism in derivatization with MSTFA. From Villas-Boas et al. 2011

1.5 Lipidomics

Biological systems include molecular lipid species belonging to several different lipid classes, which together are called the lipidome of an organism[94] (Fig. 1.9). Each molecular lipid species has biological properties that strongly depend on its chemical structure. In general, the main roles of lipids in cellular systems include energy storage, structural functions, and cellular signalling but, as it has become increasingly evident within recent years, they also play a central role in cellular regulation processes[95]. In fact, an imbalance in the lipid system can lead to pathophysiological conditions, including diabetes, atherosclerosis, and chronic inflammation[96,97]. Lipidomics is the study of lipids metabolism on a broad scale and can be used to understand the biochemical mechanisms underlying lipids imbalances that may lead to disease states. Lipidomics has greatly advanced in recent years, largerly due to developments in mass spectrometry which is considered the method of choice for this kind of analyses[98]. In particular, the coupling of mass spectrometry with liquid chromatography increases the ionization efficiency and is regarded as better suited for quantifying low abundant and isomeric lipid species[99].

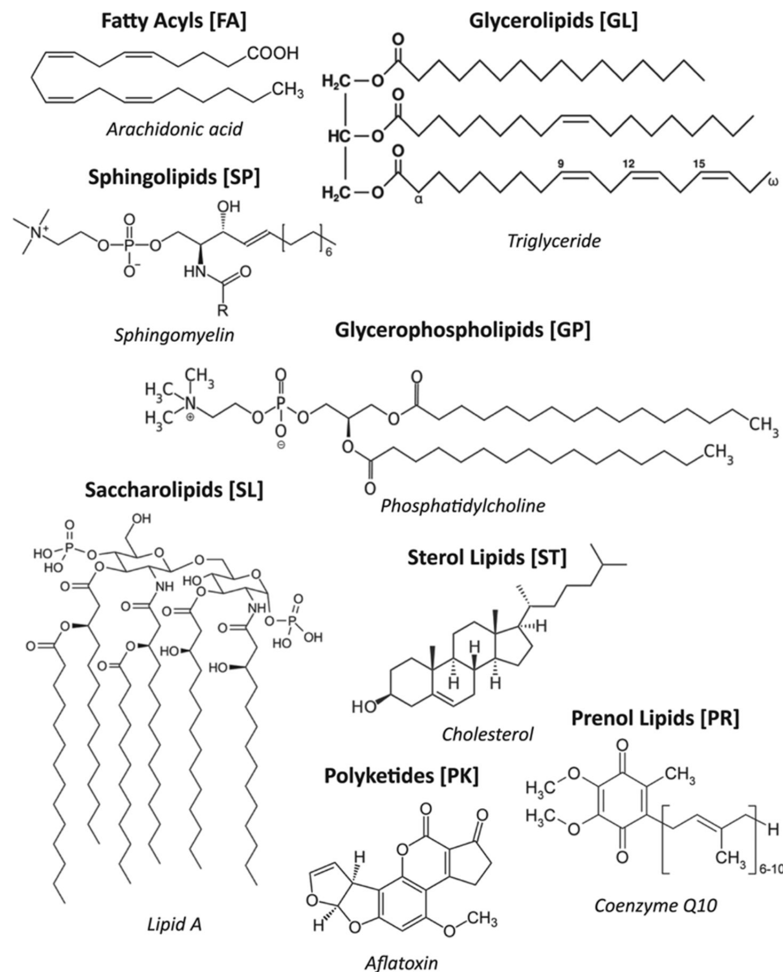


Figure 1.9: An overview of lipid classes. From Hinterwirth et al. 2014

1.6 Acylcarnitines

Acylcarnitines (AC) are esters formed through the conjugation of fatty acids with L-carnitine and are mainly employed in cellular energy metabolism pathways. In fact, their main biological function is the transportation of acyl groups from the cytosol into the mitochondria where β-oxidation occurs[100]. This process leads to the production of 7n-6 ATP molecule per AC (where ‘n’ is the number of acyl-carbons), thus providing energy to sustain cellular activity[101]. The classification of AC is based on several characteristics pertaining the chemical structure of the variable acyl moiety[102]. Usually, the first parameter taken into account is the length of the carbon chain, according to which

they can be divided into four groups:

- short-chain AC: C2-C5
- medium-chain AC: C6-C12
- long-chain AC (LCAC): C13-C20
- very long-chain AC: > C21

The fatty acid moieties can be unsaturated or saturated. Unsaturated AC are also divided into monounsaturated or polyunsaturated. Also, the cis- and trans- configuration of the fatty acid moiety can be considered. Although most AC have an aliphatic, straight-chain fatty acid moiety, some AC have branched-chains or even cyclic organic acids moieties. The fatty acid can also be substituted by several other chemical groups, such as hydroxyl- or carboxyl- groups. An overview of the possible structures of AC is reported in Fig. 1.10.

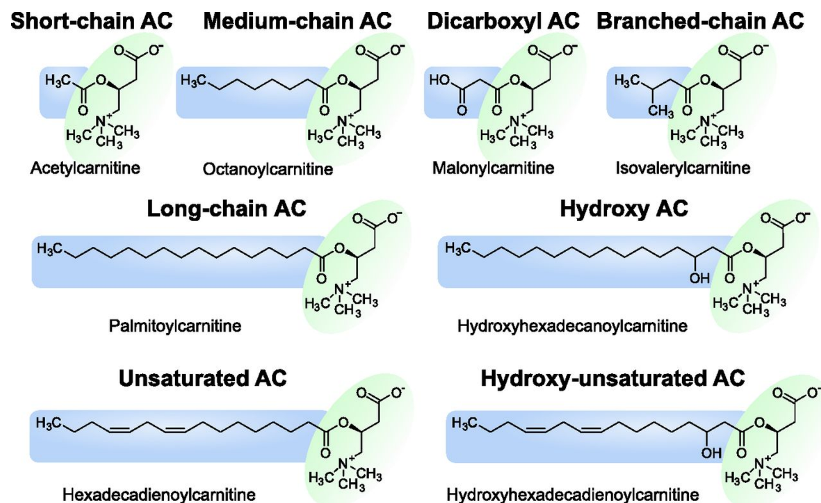


Figure 1.10: Representative structures of various acylcarnitine classes. Adapted from Dambrova et al. 2022.

Most AC are syntehsized during fatty acid metabolism. However, some can result from the degradation products of amino acids (lysine, valine, leucine, and isoleucine) or carbohydrates[103]. AC synthesis is mostly carried out by enzymes linked to the mitochondria, although peroxisomal metabolism seems to be involved as well[104].
Beside their principal role in the energetic metabolism, several studies are considering

AC as diagnostic biomarkers. For example, the concentration of plasma long-chain acylcarnitines (LCAC) is indicative of inborn errors of fatty acid oxidation and is widely used in newborn screening[105]. The interest towards AC is also growing in the field of metabolomics, as an increasing number of health conditions and diseases exhibits distinct AC profiles[102].

2 AIM OF RESEARCH

The goals of this project are:

- developing a GC-MS method to explore the metabolomic characteristics of AF to spot the metabolites or metabolite patterns that change in response to the disease or are involved in its progression;
- developing a LC-HRMS lipidomics method to focus the analysis on lipids;
- measuring key markers of inflammation to assess the link between low-grade inflammation and AF development and progression;
- studying the variation of acylcarnitines and amino acids concentrations in AF;
- determining the links between acylcarnitines, amino acids, and inflammation.

All these findings, linking bench with bedside experience, could be useful to guide the clinical management of patients with AF according to age. In particular, these data may help in the choice between rate and rhythm control therapy of the arrhythmia and could be the basis to understand the correlation between AF and frailty.

3 MATERIALS AND METHODS

3.1 Materials

- 2-mL polypropylene microcentrifuge tubes
- 2-mL glass autosampler crimp vials with micro-inserts and Teflonized seals
- glass volumetric flasks
- water (H_2O) LCMS grade
- acetonitrile (ACN) LCMS grade
- isopropanol (IPA) LCMS grade
- methanol (MeOH) LCMS grade
- chloroform ($CHCl_3$) analytical grade
- trifluoroacetic acid (TFA) LCMS grade
- formic acid (FoAc) LCMS grade
- ammonium formate
- *n*-butanol
- HCl
- extraction solvent: 3:3:2 (v/v/v) ACN/IPA/H_2O
- solution A: 2:5:2 (v/v/v) H_2O/MeOH/IPA
- QC mix (see 3.5.1)
- FAME mix (see 3.5.2)
- internal standard: succinic acid-d4 (Sigma-Aldrich), 20 ng/μL in solution A
- internal standards for LC-MS: phosphocholine (PC) 17:0 14:1 (Avanti Polar Lipids) for positive ion mode; phosphatidylinositole (PI) 17:0 14:1 (Avanti Polar Lipids) for negative ion mode
- anhydrous pyridine
- derivatization agent: D-methylhydroxylamine (MeOX)
- derivatization agent:*N*-methyl-*N*-(trimethylsilyl)trifluoracetamide (MSTFA)
- derivatization agent: *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTB-STFA)
- sodium hydroxide (NaOH) pellet (Sigma-Aldrich)

- silica gel (SiO_2) Rubin granules (Honeywell, Fluka)
- centrifuge
- orbital shaker
- magnetic stirrer

3.2 Ethical Committee approval and study subjects consent

All enrolled patients and controls gave their informed consent to participate in the study.

3.3 Enrollment, patient evaluation, and sample collection

All consecutive patients with persistent AF admitted in the Day-Hospital of the Research Unit of Medicine of Aging of the University of Florence and of the AOU Careggi to undergo elective electrical cardioversion (ECV) of the arrhythmia were enrolled in the study. The inclusion and exclusion criteria are summarized in Table 3.1. All patients were evaluated by integrating the standard clinical and instrumental cardiological visit (ECG and echocardiogram), comprehensive of the Cardio-Ankle Vascular Index (CAVI) to measure arterial stiffness, with following tools of the Geriatric Multidimensional Assessment (GMA) to describe patients' neurocognitive function, depressive symptoms, and physical performance:

- Mini-Mental State Examination (MMSE; abnormal score $\leq$ 24/30)
- Geriatric Depression Scale (GDS; abnormal score $>$ 5/15)
- Short Physical Performance Battery (SPPB; abnormal score $\leq$ 6/12)

Table 3.1: Inclusion and exclusion criteria for enrollment of patients.

Inclusion criteria	**Exclusion criteria**
AF diagnosed by ECG, ECG-Holter, or implantable device	sinus rhythm on enrollment day
age $\geq$ 60 years	-

Inclusion criteria	Exclusion criteria
consent to participate in the study	-

All patients underwent routine blood tests for hospital analysis and the results were integrated in our study (See Appendix Table 6.1).

Control subjects were enrolled among volunteers who were visited in the outpatient clinic of our Department, and who did not show any sign or symptom of cardiovascular, neoplastic, renal, and respiratory disease. A blood sample was collected from all subjects in fasting conditions using a vacutainer blood tube containing ethylenediaminetetraacetic acid (EDTA) to prevent blood from clotting. Each sample was immediately centrifuged for 15 min at 4 °C and 2,500 rpm to collect plasma, which was then stored in a refrigerator at -80 °C until analysis. The subjects were stratified in groups according to age, diagnosis of AF, and presence or absence of HF as illustrated in Table 3.2.

Table 3.2: Study groups and description.

Groups	Description
AF	all patients
AF_{only}	patients with AF but no HF
AF_{HF}	patients with AF and HF
H	all healthy controls
H_y	healthy controls aged $\leq$ 60 years (“young”)
H_o	healthy controls aged $>$ 61 years (“old”)

3.4 Subjects metadata

A database with all the available information about patients was built and updated with the information gained in the course of the study. This database was used to collect all the clinical and molecular information available about the subjects and as a basis for the statistical analyses (See Appendix Table 6.1).

3.5 Untargeted metabolomics by GC-MS

This analysis was carried out using gas chromatography coupled with mass spectrometry (GC-MS). The method is detailed below.

3.5.1 Quality Control sample

An external reference standard quality control mixture (QC mix) of 28 selected compounds was prepared following a standardized protocol[86]. Briefly, each compound was weighted and dissolved in the corresponding solvent in order to obtain the desired final concentration as detailed in Table 3.3. A stock solution was prepared by adding all compounds to a glass volumetric flask containing 25 mL of solution A and was mixed for 30 min on a magnetic stirrer. To add the QC mix to the samples, a working solution of 10 μL/mL was prepared by diluting 2.5 mL of stock solution to 10 mL using solution A.

Table 3.3: The compounds included in the QC mix.

Compound	**Concentration (mg/mL)**	**Solvent**
Pyruvate	2.0	H_2O
Alanine	2.0	H_2O
Valine	2.0	H_2O
Serine	2.0	H_2O
Nicotinic acid	2.0	H_2O
Succinic acid	2.0	H_2O
Methionine	2.0	H_2O
Aspartic acid	2.0	Solution A
4-Hydroxyproline	2.0	H_2O
Salicylic acid	2.0	H_2O
Glutamic acid	2.0	Solution A
Creatinine	2.0	H_2O
α-Ketoglutaric acid	2.0	H_2O

Compound	Concentration (mg/mL)	Solvent
N-Acetylaspartic acid	2.0	H_2O
Asparagine	2.0	H_2O
Putrescine	4.0	H_2O
Shikimic acid	2.0	H_2O
Citric acid	2.0	H_2O
Lysine	2.0	H_2O
D-(+)-Glucose	2.0	H_2O
Glucose-6-phosphate	2.0	H_2O
Arachidic acid	2.0	$CHCl_3$
Serotonin	2.0	MeOH
Adenosine	2.0	H_2O
Sucrose	2.0	H_2O
Chlorogenic acid	2.0	MeOH
α-Tocopherol	3.9	$CHCl_3$
Cholesterol	4.0	$CHCl_3$

3.5.2 Fatty acid methyl esters (FAME) mixture preparation

An internal standard mixture of fatty acid methyl esters (FAME) was prepared according to the a standardized protocol[86]. Each FAME was weighted and prepared to reach the desired final concentration (see Table 3.4).

Table 3.4: A list of the FAME employed for the study and their final concentration.

Compound	Concentration (mg/mL)
Methyl hexanoate (C06)	0.8
Methyl octanoate (C08)	0.8
Methyl nonanoate (C09)	0.8
Methyl decanoate (C10)	0.8

Compound	Concentration (mg/mL)
Methyl dodecanoate (C12)	0.8
Methyl tetradecanoate (C14)	0.8
Methyl hexadecanoate (C16)	0.8
Methyl octadecanoate (C18)	0.4
Methyl icosanoate (C20)	0.4
Mehtyl docosanoate (C22)	0.4
Methyl tetracosanoate (C24)	0.4
Methyl hexacosanoate (C26)	0.4
Methyl octacosanoate (C28)	0.4

3.5.3 Sample preparation and GC-MS analysis

The protocol was adapted from literature[86]. Plasma samples were thawed and centrifuged at 4 °C, 12,000 *g*, for 2 min. A 30 μL plasma aliquot was transferred to a new tube where 1 mL of extraction solvent was added. Samples were vortexed for 10 sec and placed on an orbital shaker for 5 min at 4 °C. After centrifuging for 5 min at the same conditions as before, 450 μL of supernatant were transferred to a new 2 mL tube and dried under nitrogen (N_2) stream at 40 °C. Samples were resuspended in 300 μL 50:50 (v/v) ACN/H_2O at room temperature, vortexed for 10 sec, centrifuged for 5 min as before, and transferred to a new tube. Samples were then dried under N_2 stream and kept in a vacuum desiccator containing NaOH pellet and SiO_2 granules to enhance the removal of water residues. After 2 h, 25 μL of internal standard solution was added to resuspend the sample, which was then vortexed for 10 sec and centrifuged according to the protocol. The supernatant was transferred to an autosampler vial, dried under N_2 stream and let rest inside the vacuum desiccator overnight to ensure the complete removal of moisture. Two derivatization methods were tested: one with MSTFA and one using MTBSTFA, which is more suitable for efficient silylation of amino acids[106]. The day after overnight desiccation, the first step of derivatization involved adding 10 μL of MeOX to the vial and shaking the sample on an orbital shaker for 1.30 h, at 37 °C

and 700 rpm. Then, 91 μL of a mixture containing 100 μL of MSTFA and 1 μL of FAME was added and the sample was left shaking for 30 min, at 37 °C and 700 rpm. A pool of samples was prepared by adding 20 μL of five samples from each group (i.e five samples of H_y, five of AF_{only}, and so on). The steps until MeOX addition were the same for derivatization with MTBSTFA but the procedure was adapted to the different derivatizing agent by adding a mixture of 50 μL ACN, 50 μL MTBSTFA, and 1 μL FAME to each sample which was then left shaking for 1 h at 80 °C and 700 rpm. The instrument used for the analysis is an Agilent Technologies GC-MS equipped with an electron ionization (EI) source and a quadrupole mass analyzer. The GC column used is a Restek 95% dimethyl/5% diphenyl polysiloxane RTX-5MS column (30-m length, 0.25-mm internal diameter, 0.25-μm film) with 10-m empty Restek guard column. Initial temperature 60 °C for 1 min; ramp 10 °C/min to 325 °C, final hold time 10 min. Samples were run in one sequence including blanks (ACN), pools, and QCmix samples every ten experimental samples.

3.6 Lipidomics by LC-HRMS

The analysis performed in LC-MS was targeted to lipids. Briefly, 10 μL of plasma were transferred to a new tube where 30 μL of IPA + 0.08 % TFA were added. 100 ng of internal standard (PC 17:0 14:1 for positive ion mode; PI 17:0 14:1 for negative ion mode) were added to the solution. The sample was left overnight at -20 °C and the following day was centrifuged for 20 min at 4 °C and 14,000 rpm. The supernatant was collected and transferred to a glass autosampler vial and 15 μL of ACN were added to reach a final IPA concentration of 50 %. 740 μL of ACN/H_2O 60:40 + 0.1 % FoAc were added to reach a final volume of 800 μL. 10 μL were injected. Mobile phases were: A = ACN:H_2O 60:40 + 10 mM ammonium formate + 0.1 % FoAc; B = ACN:IPA 10:190 + 10 mM ammonium formate + 0.1 % FoAc.

Samples were processed in both positive and negative ion modes using a liquid chromatography platform coupled with a Linear Trap Quadrupole (LTQ)-OrbitrapTM mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The multi-step gradient used for the chromatography is detailed in Table 3.5. Data were acquired in data de-

pendent acquisition mode, with a resolution of 100,000 in positive mode and 60,000 in negative ion mode. Data acquisition and analysis were carried out using the Thermo Xcalibur software (version 2.0.7).

Table 3.5: Chromatographic conditions used for the lipidomics analysis.

Retention time (min)	Flow (μL/min)	% mobile phase B
0.0	300	10
3.0	300	10
6.0	300	30
26.0	300	60
41.5	300	100
45.0	300	100
46.0	300	10
54.0	300	10

3.7 Dried Plasma Spots (DPS)

Dried plasma spots to measure acylcarnitines and amino acids concentrations were prepared according to[107,108]. Briefly, 20 μL of plasma from each sample were spotted on filter paper (903, Schleicher & Schuell) and dried. A dried plasma spot was punched into a 1.5-mL tube and 200 mL of MeOH were added. The sample was vortexed for 20 min and dried under a N_2 stream at 50 °C. The extracted acylcarnitines and amino acids were derivatized to butyl esters with *n*-butanol and HCl (3 M) at 65 °C for 25 min. After derivatization, the sample was dried under N_2 flow at 55 °C and resuspended in 200 μL H_2O/ACN (1:1) + 0.1 % FoAc. 40 mL of the diluted sample were injected in flow injection analysis (FIA) mode for MS/MS experiments using an Applied Biosystems-Sciex (Toronto, Canada) API 3200 triple quadrupole mass spectrometer equipped with a TurboIonSpray source operated in positive ion mode with a needle potential of +5,900V and turbo gas flow of 10 L/min of air heated at 150 °C. Collision-activated dissociation (CAD) MS/MS occurred in the LINAC Q2 collision cell with 10 mTorr pressure of N_2

as collision gas. The collision energy (CE) and declustering potential (DP) were optimized for acylcarnitines and amino acids using Analyst 1.4 software. For amino acids, the DP was set at +18 V and the optimal CE was 20 eV while for acylcarnitines a DP ramp (10-55 V) and a CE ramp (35-50 eV) were needed. MS and MS/MS spectra were collected in continuous flow mode. Standards of each amino acid and acylcarnitine were prepared in a 10 ng/mL solution in H_2O/ACN (1:1) + 0.1 % FoAc and infused at 10 μL/min. Quantitation experiments were done using a series 1100 Agilent Technologies (Waldbronn, Germany) CapPump coupled to an Agilent Micro ALS autosampler, both controlled by the API 3200 data system. The mobile phase was H_2O/ACN (1:1) + 0.1 % FoAc, flow rate 30 μL/min. Chromatographic and spectral interpretation and quantitative information were obtained with the Analyst 1.1 software.

3.8 IL-6 and OPG concentration measurement

The following commercially available ELISA kits were used to measure IL-6 and OPG concentration in plasma samples:

- Human IL-6 Quantikine HS ELISA kit (R&D Systems) with assay range 0.156 - 10 pg/mL in plasma;
- Osteoprotegerin Human ELISA (BioVendor R&D) with calibration range 1.5 - 60 pmol/L.

The procedure was carried out according to the vendor protocols.

3.9 Statistical analyses

3.9.1 Metadata, citokines, acylcarnitines, and amino acids

Concentration data about acylcarnitines, amino acids, IL-6 and OPG were added to the dataset containing the study subjects metadata, and all the variables in the dataset were analyzed using IBM SPSS Statistics ver. 28 (See Appendix Table 6.1). Continuous variables are expressed as mean $\pm$ standard deviation (sd), categorical variables

as raw numbers and percentages. Normal distribution of variables was assessed with the Levene test. When the variable distribution was normal, Student-*t*-test and ANOVA were used to compare two or more groups, respectively. If the variable distribution was non-normal, Mann-Whitney U test and Kruskal-Wallis test were used to compare, respectively, two groups and more than two groups of subjects. ANOVA and Kruskal-Wallis tests were followed by Tukey's *post-hoc* test. Simple linear regression analysis models were used to explore the correlation between continuous variables, and multivariable linear regression analysis models (backward deletion method) were built to identify the factors independently associated with continuous variables. To evaluate the association between categorical variable, the χ^2 test was used, followed by the Phi or Cramer's V measures to assess the strength of the correlation between dichotomous or non-dichotomous categorical variables, respectively. A two-tailed p-value < 0.05 was set as the threshold to determine statistical significance.

3.9.2 Untargeted metabolomics and lipidomics

Data obtained from the untargeted metabolomics experiment carried out using GC-MS were preliminarly processed using MSDial ver. 4.80. Data were normalized by the internal standard and metabolites have been identified using NIST, Wiley, and Fiehn mass spectra libraries. Lipids were identified using LipidFinder[109]. After normalization, peak intensities data were exported to proceed with downstream analysis using R ver. 4.1.2. Similarly, lipidomics data were analyzed using R, with a method adapted from the one developed for GC-MS analysis. All the details about the R method are explained in the Section below (3.9.3).

3.9.3 R method details

The R packages used for the metabolomics analysis are listed in Table 3.6.

Table 3.6: The R packages used for metabolomics and lipidomics data analysis.

Package	Brief description	Reference
tidyverse	a set of packages for data science including *ggplot2* (data visualization) and *dplyr* (data manipulation)	[110]
FactoMineR	multivariate exploratory data analysis and data mining	[111]
factoextra	extract and visualize results of multivariate data analyses	[112]
mclust	Gaussian mixture modelling for model-based clustering, classification, and density estimation	[113]
cluster	cluster analysis to find groups in data	[114]
NbClust	determination of the best number of clusters in a dataset	[115]
clValid	validation of clustering results	[116]
clustree	visualization of clusterings at different resolutions	[117]
cowplot	streamilined plot theme and plot annotations for *ggplot2*	[118]
scatterplot3d	plotting 3D scatter plots	[119]
bigutilsr	utility functions for large-scale data (e.g. outlier detection, unbiased PCA projection)	[120]
kableExtra	construction of complex tables	[121]
knitr	generation of a dynamic report	[122]
flagme	fragment-level analysis of gas chromatography-mass spectrometry metabolomics data	[123]

Package	Brief description	Reference
MSnbase	manipulation, processing, and visualization of mass spectrometry data	[124]
ProtGenerics	generic infrastructure for mass spectrometry packages	[125]
xcms	LC-MS and GC-MS data analysis	[126]

Briefly, raw data were imported in R, peaks were grouped and aligned using the functions provided by the *xcms* package and the RT was adjusted with the Obiwarp method. Data were normalized by the internal standard (IS) using a function specifically developed for this purpose and scaled using the Pareto method.

For the lipidomics analysis, raw data were loaded and processed in R using the packages listed in Table 3.6. Peak detection was performed using the centWave algorithm[127], with a signal-to-noise threshold set to 0, allowing 1 ppm of error, and setting the peakwidth to 10-80. The m/z center of the chromatographic peak was calculated with the *wMean* function which calculates the intensity weighted mean of the peak's m/z values. The correct integration of the internal standard was checked. After peak picking, peak alignment and retention time alignment was performed across samples using the Obiwarp method[128]. The same pre-processing methods were applied for both positive and negative ion modes and, at the end of the processing, the final files were merged into one containing results from both ionization modes to allow for a comprehensive downstream analysis. Data were visually inspected using multivariate statistical tools like Principal Component Analysis (PCA) which was also informative for the detection of outliers in the datasets. Outliers were further determined using the Local Outlier Factor (LOF) algorithm and then removed from the dataset. A cluster analysis was performed to determine unsupervised clustering of the groups using k-means clustering. The most suitable number of clusters to be used in the analyses was determined with the Silhouette method[129]. In order to shed light on what characterstics drive the clustering of the groups, the clusters obtained from the cluster analysis were analyzed using IBM SPSS Statistics ver. 28 interrogating the

database containing the metadata of the subjects enrolled in the study. To compare variables between clusters, Student-*t*-test or Mann-Whitney U test were used in case of variables that were normally or non-normally distributed, respectively.

4 RESULTS

4.1 Study population

Fifty patients with persistent AF were enrolled for the study and 22 subjects met the criteria to be included in the control group. The characteristics of each group are illustrated in Table 4.1. The AF and H groups have comparable ages ($p = 0.055$) while the percentage of women in the H group is higher than the AF group ($p = 0.012$). Age distribution across the dataset is shown in Fig. 4.1.

Table 4.1: Mean age $\pm$ standard deviation (sd), number of subjects (N), and gender representation in each study group.

Groups	**Age (mean $\pm$ sd)**	**N**	**women (%)**
AF	76 ± 6	50	32.0
AF_{only}	76 ± 6	23	30.4
AF_{HF}	76 ± 6	27	33.3
H	70 ± 14	22	63.6
H_y	56 ± 4	8	62.5
H_o	79 ± 10	14	64.3

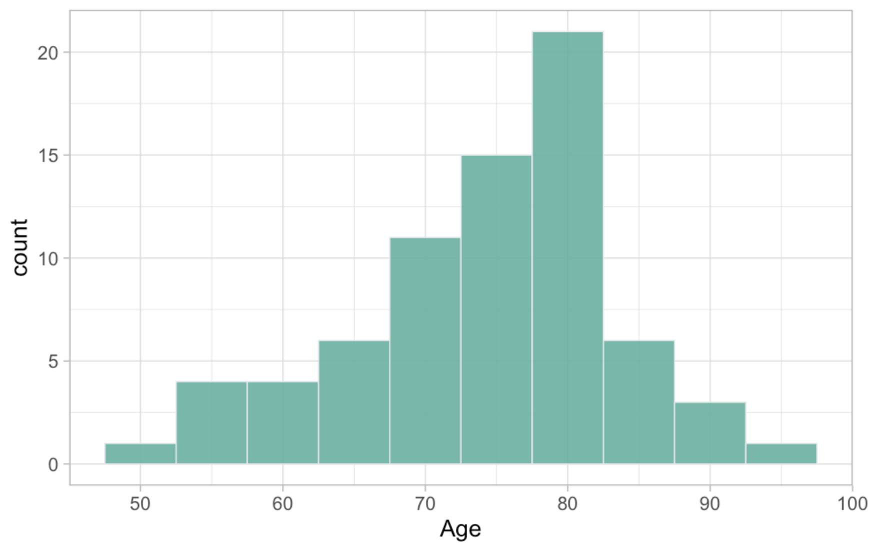


Figure 4.1: Age distribution of study subjects

4.2 Metadata analysis

From the comparison between healthy subjects (H) and patients (AF) and the analysis of the differences between the four subgroups (H_y, H_o, AF_{only}, and AF_{HF}), the CHA_2DS_2-VASc score differed both between H and AF ($p < 0.001$) and among the four groups increasing according to age and disease complication ($p < 0.001$) (Fig. 4.2).

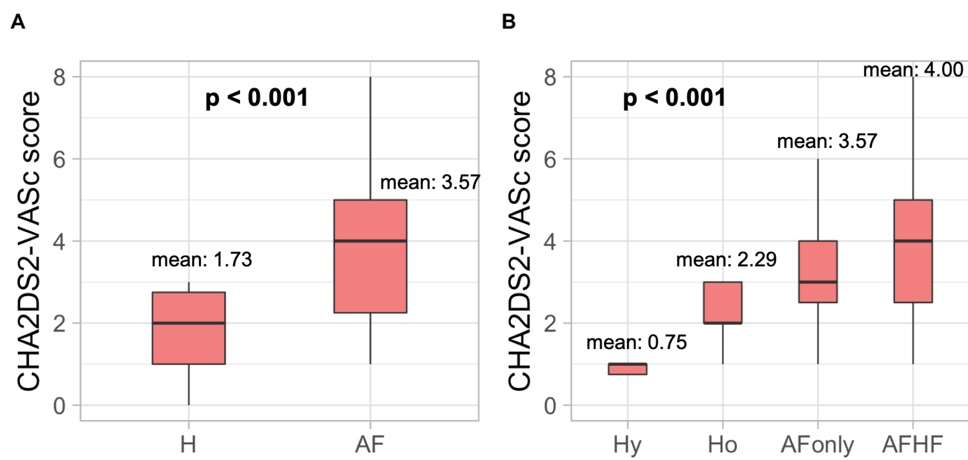


Figure 4.2: Boxplots showing the significant difference in CHA2DS2-VASc score between H and AF groups (A) and among the four subgroups (B)

The metadata available for the subjects included in the AF group (see Table Appendix 6.1) were tested to assess the differences between the subgroups AF_{only} and AF_{HF}. Left ventricular ejection fraction (LVEF) resulted significantly lower in patients with HF (p = 0.014) as well as the BMI (p = 0.030). Differences in end-diastolic (EDD) and end-systolic diameters (ESD) also emerged (EDD p = 0.018; ESD p = 0.022). Variations in Ca^{2+} levels were detected, with subjects without HF having a slightly higher concentration of circulating Ca^{2+} (p = 0.043). NT-proBNP levels also differed between the two groups, with AF_{HF} subjects having a significantly higher level of the peptide (p = 0.019) (Fig. 4.3).

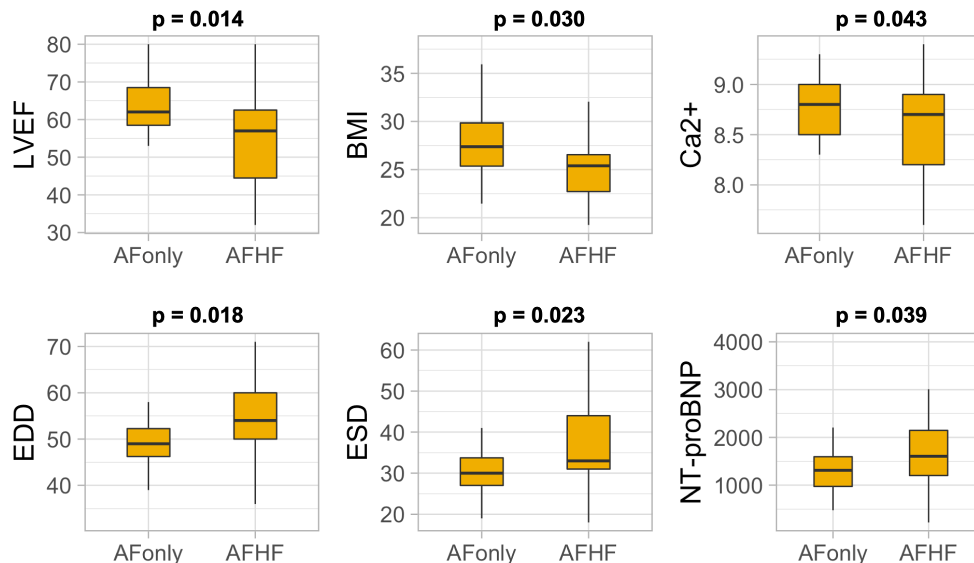


Figure 4.3: Significant differences that emerged between AF only and AF HF groups.

4.3 CHA_2DS_2-VASc score and Geriatric Multidimensional Assessment

Together with the analyses carried out on the main dataset, the plasma of 134 patients was used to measure additional IL-6 and OPG levels to study the relationship between low-grade inflammation and frailty, measured by the CHA_2DS_2-VASc score and the questionnaires of the Geriatric Multidimensional Assessment (GMA). Overall, inflammatory mediators in 93 (69.4 %) of the 134 patients enrolled in this study protocol were measured. Mean age was 77 $\pm$ 8 years (men: 64.5%; BMI: 26.7 $\pm$ 4.0

Kg/m^2). The most frequently observed comorbid condition was hypertension (82.8%). The prevalence of congestive heart failure, diabetes, stroke or transient ischemic attack and vascular diseases was 29.0, 16.1, 12.9 and 29.0%, respectively. Erythrocytes sedimentation rate and C reactive protein concentration was below the abnormal values in more than 70% and 80% of cases. Subjects with a CHA_2DS_2-VASc score > 4 (N = 51, 54.8 %), when compared with those with a score < 3 (N = 42, 45.2 %), had higher values of IL-6 and OPG (retrospective power for the comparisons: 77.2 and 81.9 %, respectively). Uric acid, iron, ferritin, hemoglobin concentration, and the glomerular filtration rate (GFR) were lower in patients with a CHA_2DS_2-VASc score > 4. In linear regression models, IL-6 maintained its statistical association with the CHA_2DS_2-VASc score, while OPG only approached statistical significance (β = 0.32 $\pm$ 0.17, R = 0.216, p = 0.061). When separately evaluating the different components of the CHA_2DS_2-VASc, we found that IL-6 concentration was higher in patients > 75 years (4.1 $\pm$ 3.0 vs. 3.2 $\pm$ 3.0 pg/mL, p = 0.042) and in those with diabetes (4.9 $\pm$ 3.0 vs. 3.6 $\pm$ 3.0 pg/mL, p = 0.039) and vascular diseases (4.8 $\pm$ 3.3 vs. 3.4 $\pm$ 2.8 pg/mL, p = 0.044). IL-6 maintained an association with age also using a linear regression analysis model (β = 0.10 $\pm$ 0.04, R = 0.269, p = 0.011). OPG was significantly higher only in patients with vascular diseases (5.3 $\pm$ 2.9 vs. 3.7 $\pm$ 2.1 pmol/L, p = 0.035); no differences were observed for the other components of the CHA_2DS_2-VASc score. When studying the tools of the GMA, IL-6 showed an inverse association with SPPB; no correlation was found with MMSE and GDS scores. SPPB, as previously found, was inversely related to the CHA_2DS_2-VASc (β = -0.75 $\pm$ 0.15, R = 0.461; $p < 0.001$) and the GDS ($p < 0.001$) scores. Among the other variables, IL-6 was associated with the concentration of iron and uric acid. In multivariate analysis (R = 0.501; $p < 0.001$), only SPPB (β = -0.42 $\pm$ 0.12; p = 0.001) and iron (β = -0.04 $\pm$ 0.01; $p < 0.001$) maintained their association with the cytokine levels, while CHA_2DS_2-VASc score (p = 0.324) and uric acid (p = 0.122) were deleted from the model. OPG did not show any correlation with GMA tools. Higher levels of hemoglobin corresponded to lower levels of OPG (β = -0.36 $\pm$ 0.16, R = 0.247; p = 0.032).

4.4 Untargeted metabolomics by GC-MS

The efficacy of the two derivatization methods was assessed by evaluating the reproducibility of each method and comparing the intensity of peaks obtained in samples derivatized with MSTFA with the intensity of peaks of TBDMS derivatives. Both derivatization methods worked for most metabolites and, as expected, MTBSTFA derivatization resulted more efficient in derivatizing amino acids. However, amino acids were also detected as TMS derivatives and, overall, MSTFA yielded better results in terms of peak intensity, sample reproducibility, as well as reaction and was thus selected as the elective derivatization method for our analysis. Overall, 67 samples, 44 AF and 22 H, were analyzed. The internal standard (IS) peak, corresponding to succinic acid-d4, was detected in all samples the RT range 570-690 and at m/z range 250.1-252.1. A total ion current (TIC) profile is shown in Fig. 4.4.

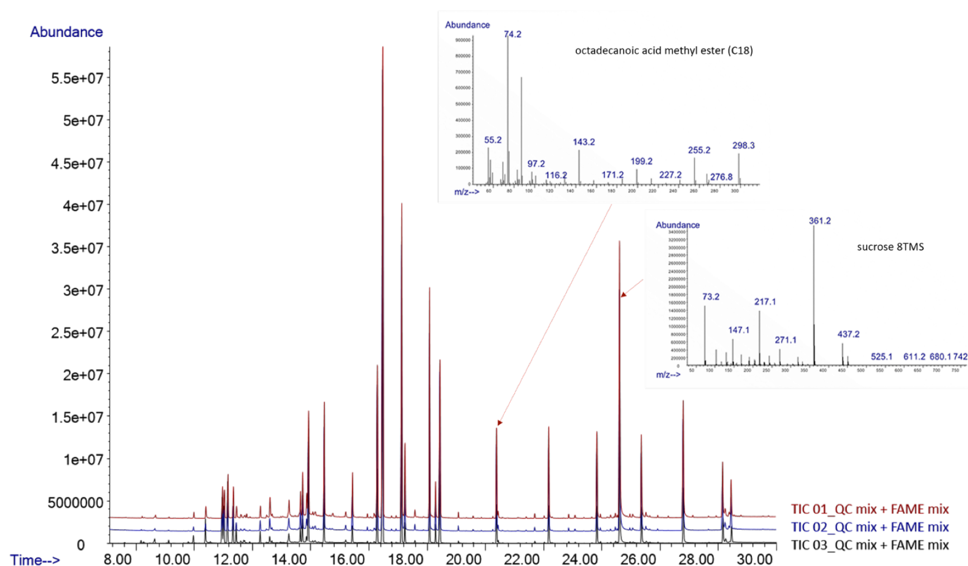
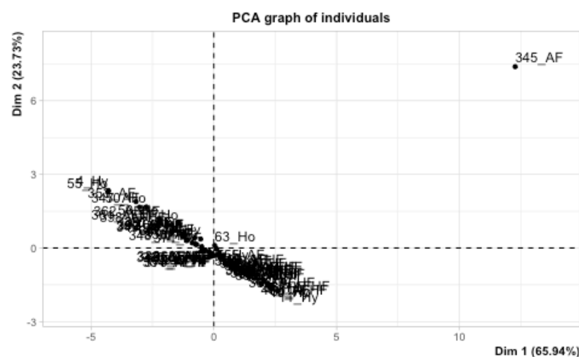


Figure 4.4: Total ion current of a metabolomic sample repeated three times to show reproducibility. The spectra of two identified molecules are shown in the inserts.

A preliminary data exploration using PCA allowed for the identification of sample 345 AF as an outlier. This was also confirmed by the Local Outlier Factor (LOF) outlier detection method applied to the dataset (Fig. 4.5) .



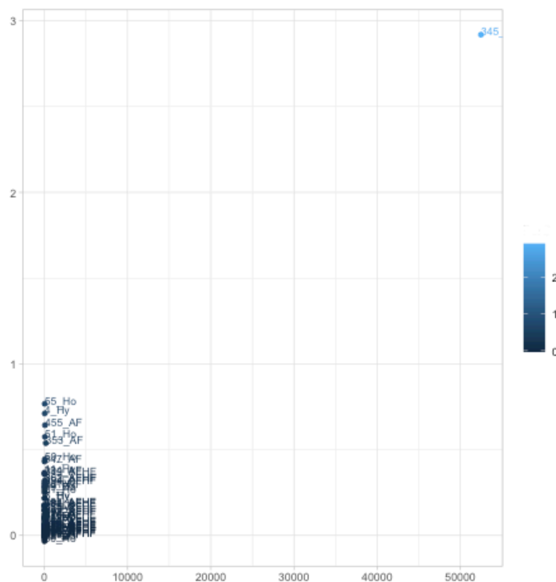


Figure 4.5: Above: A Principal Component Analysis (PCA) showing the presence of an outlier sample. Below: The outlier highlighted by the Local Outlier Factor (LOF) outlier detection method.

After removing the outlier from the dataset, a new PCA was performed to visually inspect the data. The PCA successfully separated the QCmix samples and the blank samples (ACN) from the plasma samples as highlighted in Fig. 4.6 and pool samples are grouped within the other plasma samples.

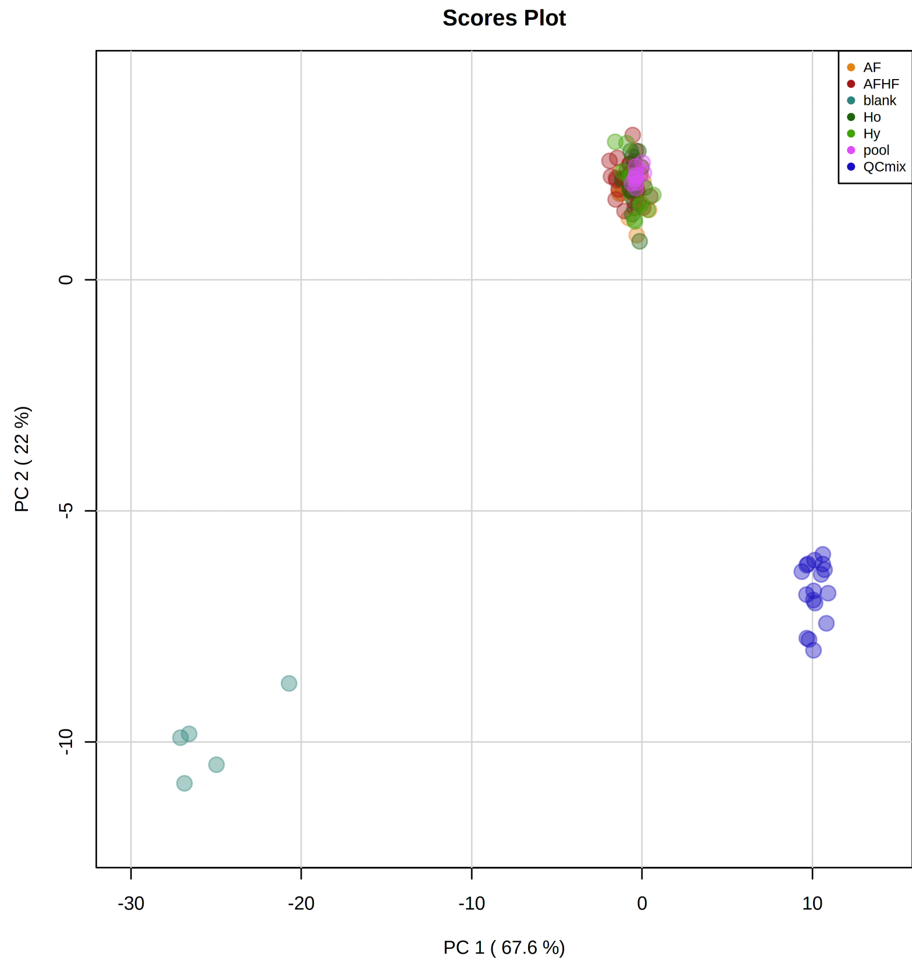


Figure 4.6: PCA performed including QCmix samples, blanks, and plasma samples.

After removing the QCmix, blank, and pool samples from the dataset, a PCA was performed again to see if a separation between the study groups was detectable. Based on the screeplot (Fig. 4.7), the principal components that explain most of the variance are PC1 and PC2, which are displayed in the PCA plot in Fig. 4.7, from which it emerges that no clear separation between the four groups is appreciable with this method.

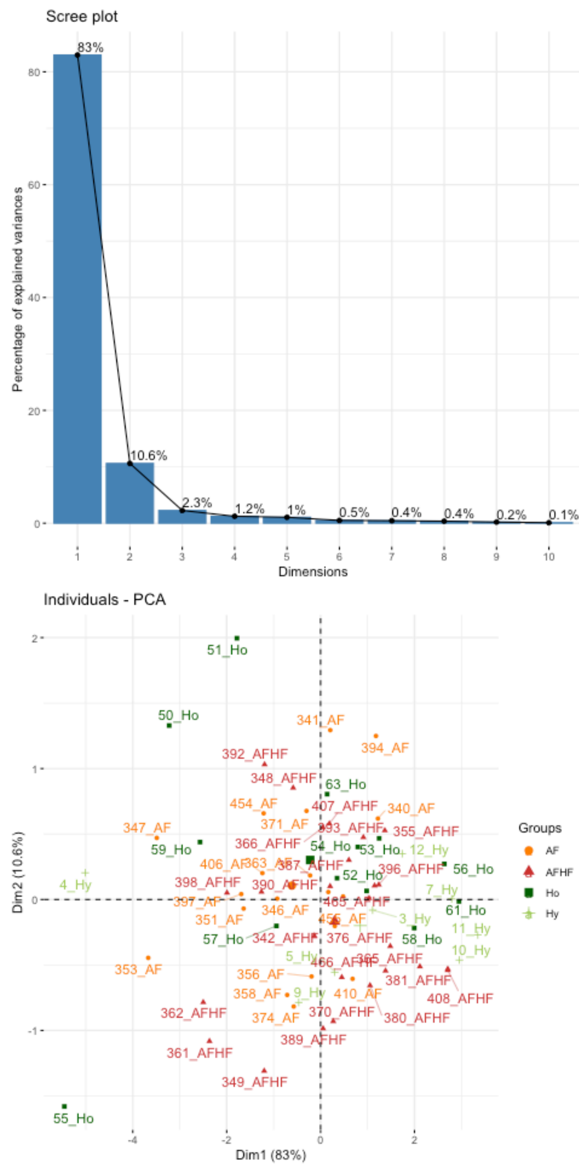


Figure 4.7: Above: Screeplot indicating the principal components (PCs) that explain most of the variance. Below: PCA of samples.

The separation is not detectable even when dividing the dataset in two groups, H and AF as shown in Fig. 4.8.

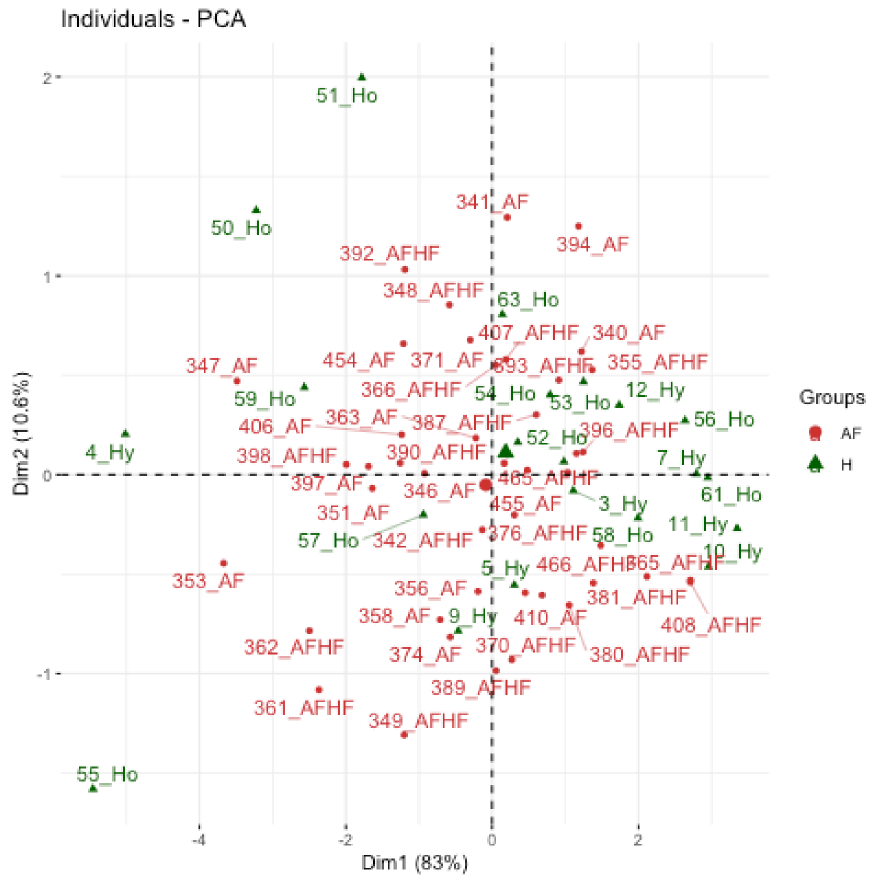


Figure 4.8: PCA of samples divided into the two main groups, H and AF.

The same approach was followed to visually examine the distribution of samples within the AF group. No clear separation between AF_{only} and AF_{HF} subjects is appreciable (Fig. 4.9).

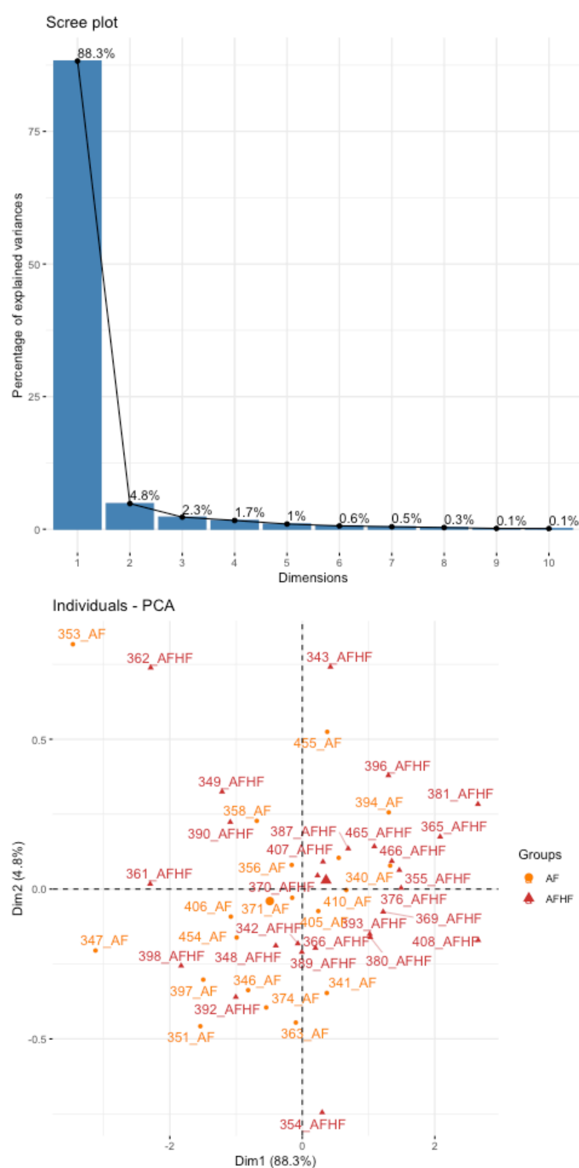


Figure 4.9: Above: Screeplot indicating the principal components (PCs) that explain most of the variance. Below: PCA of AF samples.

4.4.1 Cluster analysis of metabolomics data

Following this result, an unsupervised cluster analysis was performed to determine which characteristics are relevant to differentiate groups within the dataset. The Silhouette method for determining the correct number of clusters to use in the analysis suggested to set two groups to maximize the variance between clusters (Fig. 4.10).

The k-means clustering algorithm suggested the grouping shown in Fig. 4.10.

Figure 4.10: Above: Silhouette analysis to detect the best number of clusters to be used in cluster analysis. Below: results from the k-means clustering algorithm.

The groups determined by the cluster analysis were set as independent variables and metadata of patients and controls were used as dependent variables. The two clusters differed only based on the weight and BMI of subjects, with Cluster 1 grouping subjects with lower BMI and weight (Table 4.2). None of the categorical variables analyzed with the χ^2 test resulted to be significant.

Table 4.2: Analysis of clusters and metadata (variables) of patients and controls using k-means clustering. Values are expressed as means $\pm$ sd when a parametric test was used, whereas mean ranks are specified when a non-parametric test was employed.

Variable	p-value	mean $\pm$ sd or mean rank Cluster 1 (N = 17)	mean $\pm$ sd or mean rank Cluster 2 (N = 35)
Weight	0.032	74.10 ± 11.86	81.76 ± 11.48
BMI	0.046	23.57	32.53

The same data analysis was performed with only the samples of patients, to assess the differences within the AF group. Interestingly, one of the two clusters included subjects with lower SPPB score, higher CHA_2DS_2-VASc score, and higher IL-6 levels (Table 4.3 and Fig. 4.11)

Table 4.3: Analysis of clusters and metadata (variables) about AF patients using k-means clustering. Values are expressed as mean ranks as a non-parametric test was employed.

Variable	p-value	mean rank Cluster 1 (N = 16)	mean rank Cluster 2 (N = 29)
SPPB	0.009	19.24	29.81
CHA_2DS_2-VASc score	0.007	26.86	16.00
IL-6	0.002	25.96	16.44

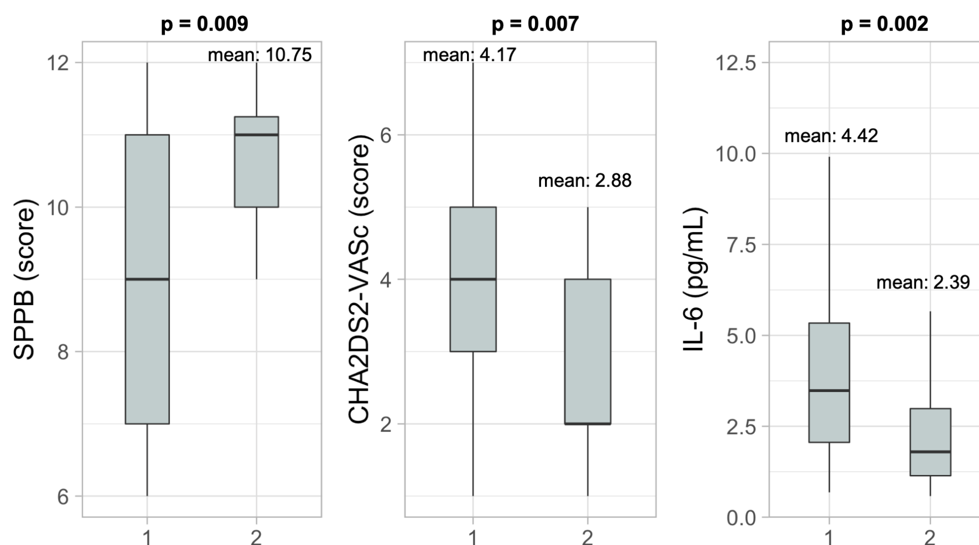


Figure 4.11: Boxplots showing the differences between the two clusters determined by k-means clustering.

The χ^2 test highlighted that Cluster 2 includes more subjects having dyslipidemia (p = 0.011). A detailed table of the clusters obtained with k-means clustering can be found in the Appendix Tables 6.3 and 6.4.

4.5 Lipidomics analysis by LC-HRMS

72 samples, including 46 AF and 26 H, were run in both positive and negative ion modes. PC 17:0 14:1 (m/z 718.5381) was used as internal standard for the positive mode and PI 17:0 14:1 (m/z 793.4873) for negative mode. Data were visually inspected and peaks within the retention time window 20 - 2280 s were selected for downstream analysis. The extracted features were searched in Lipid Finder and several classes of lipids were identified (Fig. 4.12)

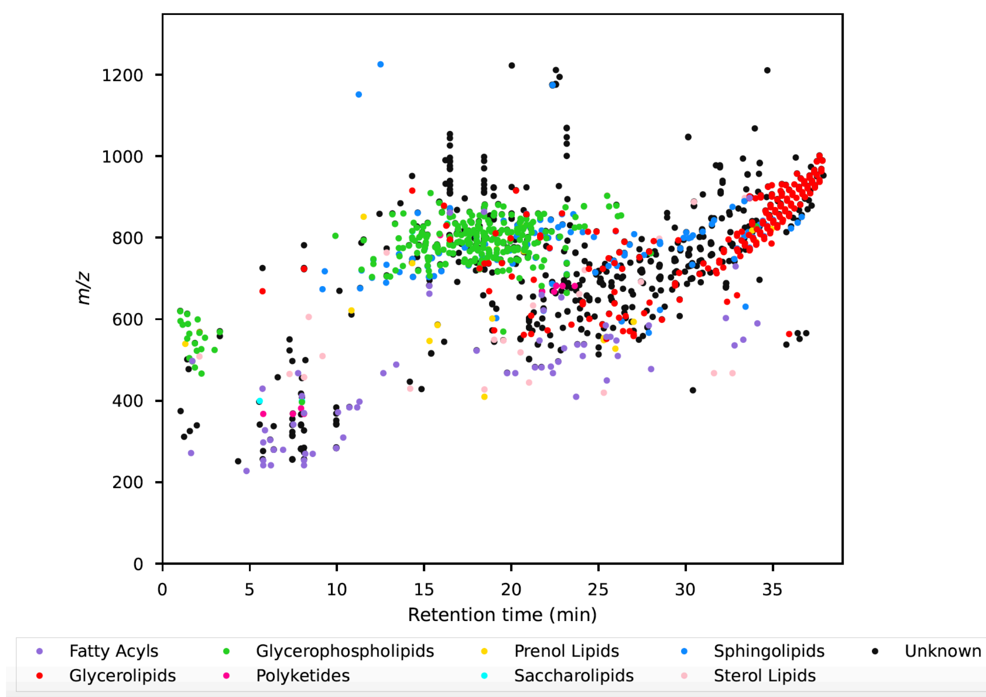


Figure 4.12: Classes of lipids present in the samples and identified through Lipid Finder.

Visual inspection of the dataset with PCA followed by Local Outlier Factor determined the following samples as outliers: 352 AF, 59 H_o, 60 H_o, 61 H_o, 62 H_o, 63 H_o.

After removing the outliers from the dataset, a PCA was performed to see if any separation occurs in the lipidomic profile of the study groups. PC1 and PC2 explain most of the variance and thus have been used for plotting the PCA.

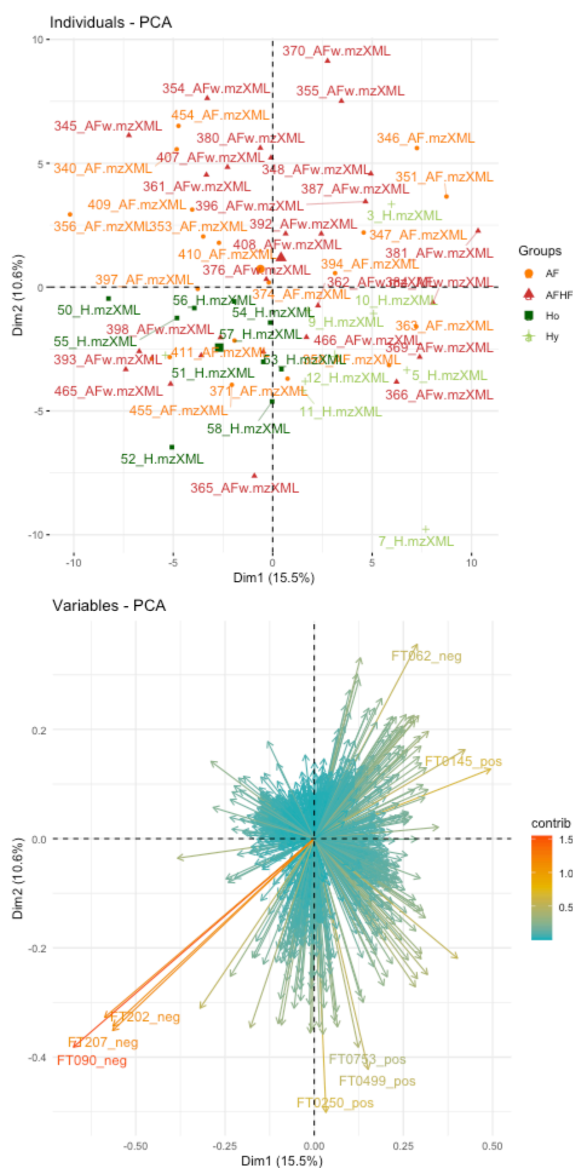


Figure 4.13: Above: PCA of lipidomics samples. Below: Loading plot showing the features that drive the separation.

Although no clear separation can be appreciated among groups, H_o samples seem to be clustered in the lower left quadrant of the PCA biplot while H_y mostly group in the lower right quadrant. Based on this result, a loading plot was executed to detect which features drive the separation toward the region where H samples seem to cluster.

4.6 Acylcarnitines and amino acids

A detailed list of the compounds that were measured with the dried plasma spot method is summarized in Table 4.4.

Table 4.4: Acylcarnitines and amino acids measured for this study.

Acylcarnitines	**Amino acids**
free carnitine (C0)	alanine (Ala)
acetylcarnitine (CAR 2:0)	valine (Val)
propanoylcarnitine (CAR 3:0)	leucine/isoleucine (Xle)
propenoylcarnitine (CAR 3:1)	methionine (Met)
butyrylcarnitine (CAR 4:0)	phenilalanine (Phe)
hydroxybutyrylcarnitine (CAR 4:0 OH)	tyrosine (Tyr)
isovalerylcarnitine (CAR 5:0)	aspartic acid (Asp)
ethylacryloylcarnitine (CAR 5:1)	glutamic acid (Glu)
hexanoylcarnitine (CAR 6:0)	ornithine (Orn)
hexenoylcarnitine (CAR 6:1)	arginine (Arg)
octanoylcarnitine (CAR 8:0)	citrulline (Cit)
octenoylcarnitine (CAR 8:1)	glycine (Gly)
decanoylcarnitine (CAR 10:0)	
decenoylcarnitine (CAR 10:1)	
decadienoylcarnitine (CAR 10:2)	
hydroxydecanoylcarnitine (CAR 10:0 OH)	
dodecanoylcarnitine (CAR 12:0)	
dodecenoylcarnitine (CAR 12:1)	
hydroxydodecenoylcarnitine (CAR 12:0 OH)	
myristoylcarnitine (CAR 14:0)	
tetradecenoylcarnitine (CAR 14:1)	
tetradecadienoylcarnitine (CAR 14:2)	
hydroxymyristoylcarnitine (CAR 14:0 OH)	
palmitoylcarnitine (CAR 16:0)	

Acylcarnitines	Amino acids
palmitoleoylcarnitine (CAR 16:1)	
hydroxypalmitoylcarnitine (CAR 16:0 OH)	
hydroxypalmitoleoylcarnitine (CAR 16:1 OH)	
stearoylcarnitine (CAR 18:0)	
octadecenoylcarnitine (CAR 18:1)	
octadecadienoylcarnitine (CAR 18:2)	
hydroxystearoylcarnitine (CAR 18:0 OH)	
hydroxyoctadecenoylcarnitine (CAR 18:1 OH)	
hydroxyoctadecadienoylcarnitine (CAR 18:2 OH)	

The plasmatic concentration of acylcarnitines and amino acids was compared between groups. AF subjects resulted to have lower Asp, Arg, and Gly compared to controls (Asp $p = 0.017$; Arg see details below; Gly $p = 0.002$) and higher concentrations of CAR 3:1 and CAR 5:0 (CAR 3:1 $p = 0.002$; CAR 5:0 $p = 0.037$) (Fig. 4.14).

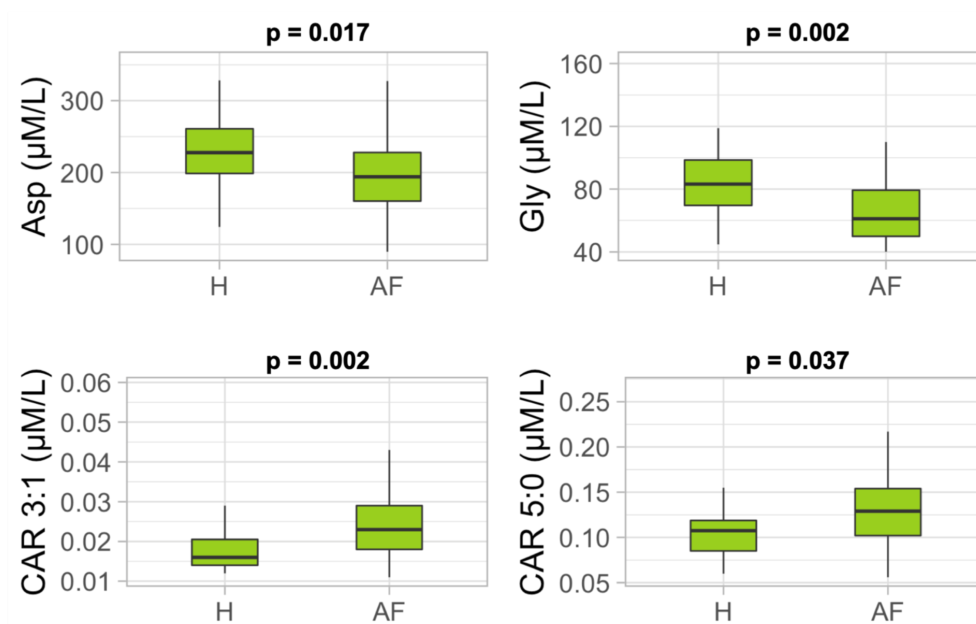


Figure 4.14: Significant differences in amino acids and acylcarnitines concentrations between AF patients and H controls.

When analyzing the four subgroups, Arg and Gly concentrations decreased significantly (Arg see details below; Gly p = 0.011) (Fig. 4.15). The same analyses were carried out on patients, to investigate the differences between AF_{only} and AF_{HF}. Both CAR 12:1 and CAR 14:1 increased in patients with HF (CAR 12:1 p = 0.031; CAR 14:1 p = 0.035) (Fig. 4.16).

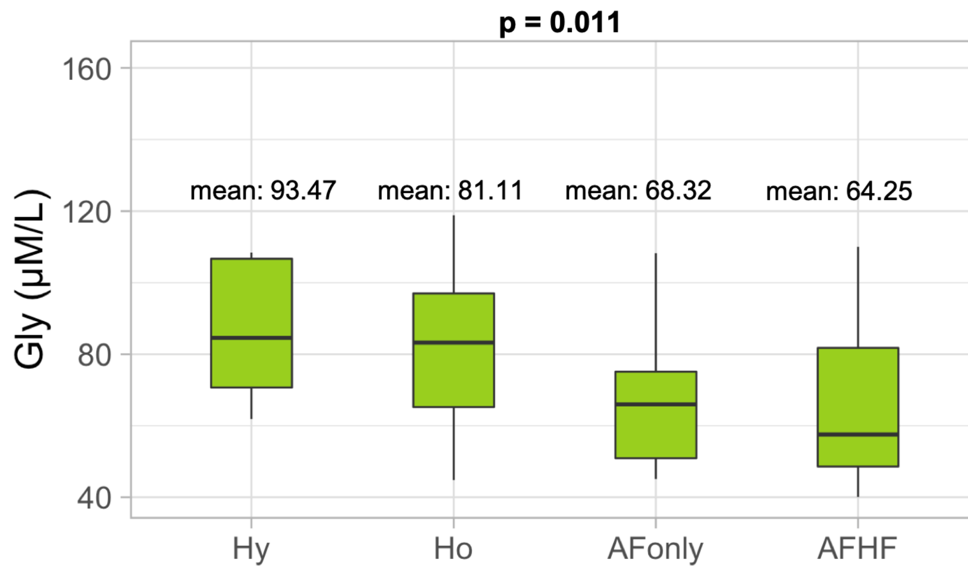


Figure 4.15: Decreasing trend in Gly concentration.

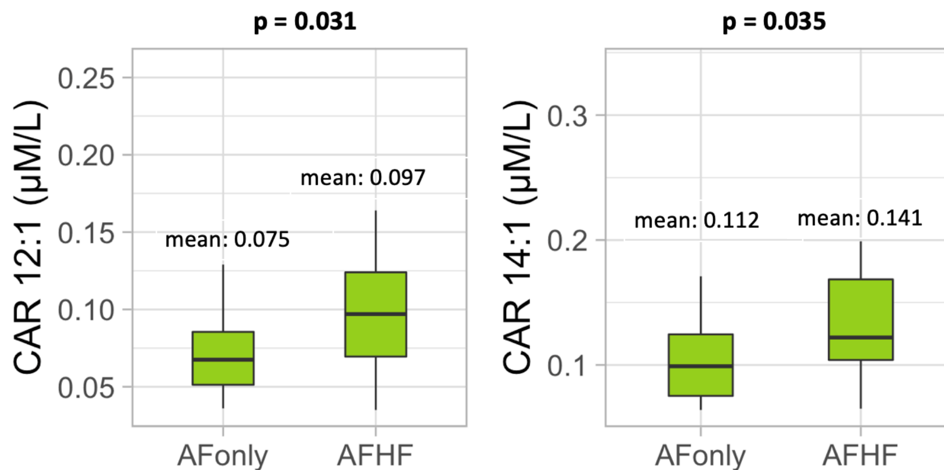


Figure 4.16: Increase in medium- long-chain acylcarnitines in patients with HF.

4.6.1 Arginine

Among the amino acids measured in this project, arginine (Arg) was analyzed in depth as it is an established marker of endothelial function[130]. The amino acid levels were significantly lower when AF was present (56 $\pm$ 17 vs. 71 $\pm$ 23 μmol/L; p = 0.003); a post-hoc analysis showed a statistically significant difference between H_y and patients; no differences were observed between H_y and H_o individuals and patients (Fig. 4.17). A multivariate linear regression analysis model with, at each step, backward deletion of the variable showing the lowest statistical association (R = 0.547, p $<$ 0.001), found an inverse association linking Arg concentration to age (β = -0.93 $\pm$ 0.22; p $<$ 0.001) and to the presence of AF (β = -9.71 $\pm$ 4.58; p = 0.038), whereas sex was deleted from the model (p = 0.666).

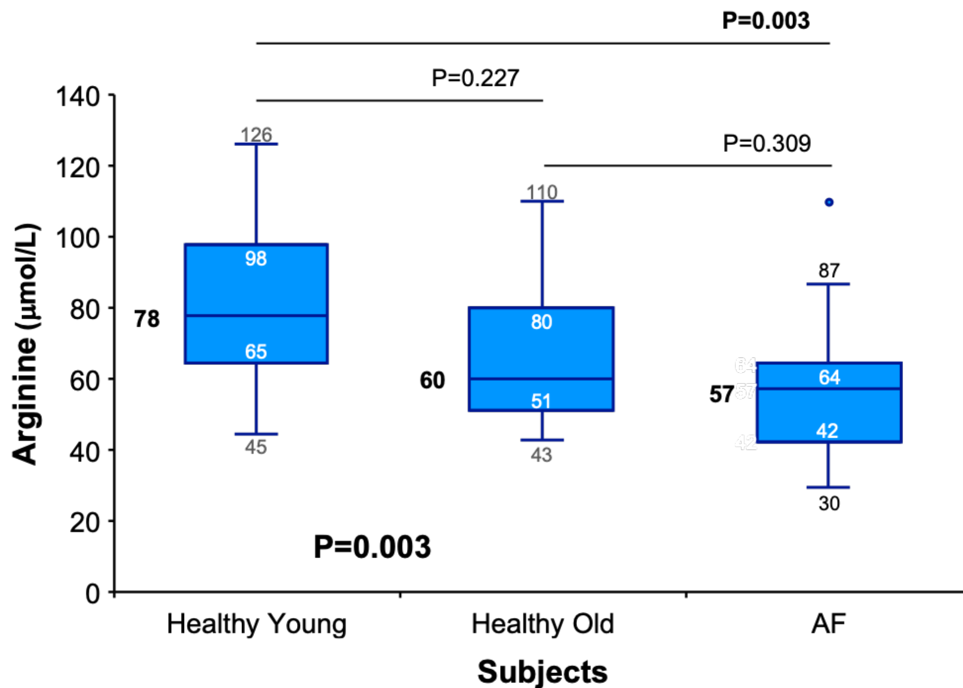


Figure 4.17: Arginine concentration in the groups

When limiting the analysis to patients with the arrhythmia, an inverse relation linking Arg and age was observed. No association of the amino acid concentration was found with sex, BMI, heart rate, hypertension, diabetes, dyslipidemia, chronical renal faiure,

LVEF, CAD, cerebrovascular disease, and peripheral artery disease. Accordingly, also the $CHAD_2DS_2$-VASc score was unrelated to Arg concentration, as well as MMSE, GDS, and SPPB. Arg levels were associated with white blood cells count, and with iron and glutamic-pyruvic transaminase concentration. Interestingly, IL-6 did not show any correlation with Arg, as observed for arterial stiffness, and the most important cardiovascular drugs. Citrulline levels were directly related to Arg concentration (β = 0.85 $\pm$ 0.16; R = 0.619; p $<$ 0.001). A multivariate linear regression analysis model (R = 0.626, p $<$ 0.001) confirmed the age-related Arg decrease in patients with AF (β = -0.91 $\pm$ 0.37; p = 0.019). Also, it showed the inverse association between Arg and iron levels (β = -0.19 $\pm$ 0.07; p = 0.009) and the direct correlation between the amino acid and the glutamic-pyruvic transaminase concentration (β = 0.21 $\pm$ 0.07; p = 0.037), whereas white blood cells count was deleted from the model (p = 0.318).

4.7 IL-6

IL-6 concentration was significantly higher in patients with AF when compared to the control group (p = 0.011) (Fig. 4.18). When the control group was stratified according to age, an increase in IL-6 concentration was detected with increasing age and in presence of AF (Fig. 4.19). IL-6 is higher also in presence of hyperuricemia when compared to patients with normal uric acid levels (p = 0.004).

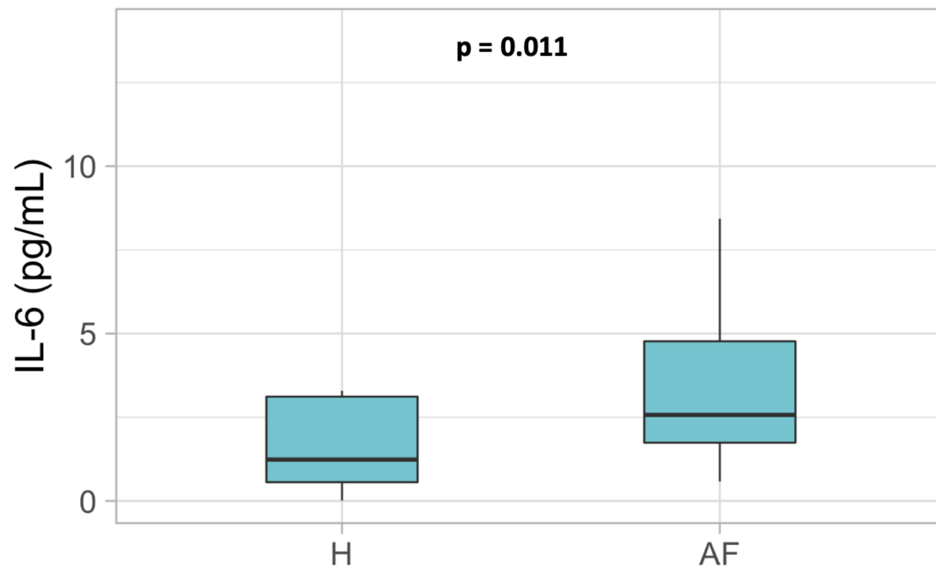


Figure 4.18: IL-6 in controls and AF patients.

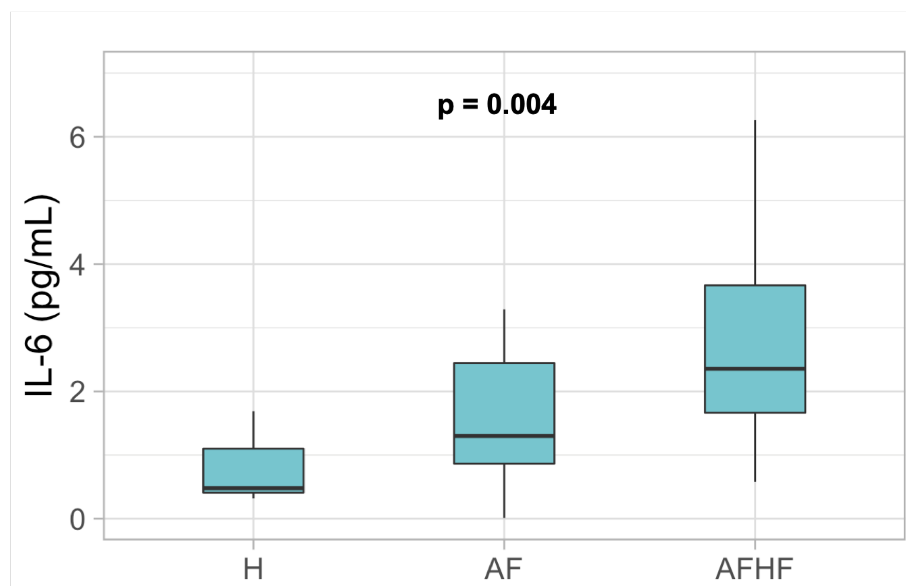


Figure 4.19: Trend of IL-6 concentration.

A simple linear regression model showed that IL-6 was inversely related to SPPB. The overall regression was statistically significant (R^2 = 0.275, $p < 0.001$). Similarly, a lower MMSE score was associated with higher IL-6 concentration (R^2 = 0.129, p = 0.012). No significant association was found between IL-6 and the GDS score. A

significant correlation was found when CRP was the independent variable (R^2 = 0.268, p = 0.001), as with hyperuricemia (R^2 = 0.223, $p < 0.001$). An association between IL-6 and the natriuretic peptide NT-proBNP concentrations was also found (R^2 = 0.217, p = 0.003). No association between the cytokine levels and CAVI was detected. Results are summarized in Table 4.5.

A multiple linear regression model using the backward deletion method was built including variables significantly associated with IL-6 levels as independent variables in univariate analysis. In the final step (R = 0.615, $p < 0.001$), the citokine concentration was correlated directly with uric acid levels ($\beta \pm$ SE = 0.056 $\pm$ 0.23, p = 0.021) and inversely with SPPB score ($\beta \pm$ SE = -0.78 $\pm$ 0.17, 95% CI = -1.13/-0.43). Age, sex, chronic renal and chronic heart failure, diabetes, and MMSE score were deleted from the model.

Table 4.5: Significant associations between IL-6 and patients' clinical characteristics (univariate analysis).

Variable	$\beta \pm$ SE	R	p-value	95 % CI for β
SPPB - total (score)	-0.744 $\pm$ 0.176	0.525	$<$ 0.001	-1.098; -0.390
MMSE (score)	-0.500 $\pm$ 0.192	0.358	0.012	-0.887; -0.114
GDS (score)	-	-	ns	-
CRP (mg/L)	0.274 $\pm$ 0.079	0.518	0.001	0.114; 0.434
Hyperuricemia (y/n)	3.108 $\pm$ 0.846	0.472	$<$ 0.001	1.407; 4.810
NT-proBNP (pg/mL)	0.001 $\pm$ 0.000	0.465	0.003	0.000; 0.001
RCAVI	-	-	ns	-
LCAVI	-	-	ns	-

Medium- long-chain acylcarnitine concentrations also significantly predicted IL-6 levels as illustrated in Table 4.6.

Table 4.6: Linear regression analysis for IL-6 with acylcarnitines

Variable	$\beta \pm$ SE	R	p-value	95 % CI for β
CAR 8:1	65.515 $\pm$ 28.986	0.316	0.029	7.168; 123.861
CAR 12:1	31.737 $\pm$ 9.390	0.446	0.001	12.835; 50.638
CAR 12:0 OH	53.085 $\pm$ 23.673	0.314	0.030	5.434; 100.736
CAR 14:0	38.911 $\pm$ 15.936	0.339	0.019	6.834; 70.988
CAR 14:1	15.188 $\pm$ 6.942	0.307	0.034	1.215; 29.162
CAR 14:0 OH	161.143 $\pm$ 60.919	0.363	0.011	38.519; 283.766
CAR 18:1	19.927 $\pm$ 8.239	0.336	0.020	3.344; 36.511
CAR 18 OH	161.545 $\pm$ 68.771	0.327	0.023	23.115; 299.975

The same analyses were carried out using OPG instead of IL-6 and no parameter resulted to be significantly associated with this citokine.

5 DISCUSSION AND CONCLUSIONS

The results we have just shown demonstrate that, when clustering metabolomic profiles of older patients with persistent AF and healthy subjects, a difference in their body size emerges; furthermore, when analyzing only patients with the arrhythmia, it is possible to identify three clinical-laboratory characteristics, namely, the CHA_2DS_2-VASc score, the SPPB score and the IL-6 levels, that allow to define two clusters of subjects with different patterns at the untargeted metabolomic analysis. IL-6 concentration is inversely associated with SPPB score and directly correlated with uric acid and medium- long-chain acylcarnitine levels, and, when studying a broader cohort of individuals, also with the CHA_2DS_2-VASc score. We also found that arginine (Arg) levels are significantly lower in AF patients, even after having adjusted for age, and we hypothesize the existence of endothelial dysfunction when arrhythmia is present.

Different metabolomic profiles by body weight or BMI when simultaneously analyzing younger healthy controls and the AF cohort can be explained by the progressive reduction of muscular mass, i.e. sarcopenia, observed after 45 years of age. In particular, in subjects equal or older than 90 years, muscular mass is 50 % lower than that usually found at younger ages. Possible explanations of this phenomenon are represented by a sedentary lifestyle, the reduced uptake of proteins and calories, and the decreased trophic action exerted by the autonomic nervous system fibers. All these changes are coupled with the reduced production of steroid hormones, mitochondrial dysfunction, the increased inflammatory burden, and the presence of oxidative damage[131]. Also, the genome can exert an influence on sarcopenia, as demonstrated by the InChianti Study, where, in the original cohort of subjects older than 65 years, 5 transcripts allowed to identify a “biologically” younger group, characterized by lower levels of IL-6 and urea nitrogen, a higher concentration of albumin and a greater strength than controls[132]. Importantly, the changes just illustrated can explain the complex interactions leading from decline in mobility, through functional impairment, to the increase in mortality[131]. Sarcopenia is also an essential component of frailty. The condition, a multi-factorial syndrome, is caused by the reduction of physiological reserve and the possibility to resist to stressful events. Hence, it can be conceived as the lack of the homeostatic capacity.

It is often discovered in older individuals, but it can be found also when other conditions, such as heart failure, chronic kidney disease, and cancer are present. Frailty is a complex and dynamic disorder. Importantly, it can lead to an increased incidence of disability, and to higher rates of hospitalization, institutionalization, and mortality. It is now thought that frailty can be in great part attributed to a process characterized by the progressive loss of ability to produce, distribute, and utilize energy[66]. Interestingly, in a survey led by the European Heart Rhythm Association (EHRA) among physicians involved in the management of arrhythmias, 72 % of the respondents thought that AF was one of the comorbidities most frequently observed in frail individuals. Also, the bradycardia-tachycardia syndrome, often associated with the arrhythmia, was thought to be often present in frail subjects[66]. The prevalence of frailty is highly variable in the most important AF studies, usually ranging between 20 and 90 %, according to the protocol which was followed (i.e., randomized trial or prospective registry), the clinical setting which was studied (i.e., hospital, outpatient clinic, and home), the specialization of the involved physicians and the geographical area, with the related culture, in which the observation takes place[133,134]. Despite the methodological and the related epidemiological differences, all experiences are concordant in showing a mortality risk 2.5 times higher in AF frail patients when compared to non-frail arrhythmic subjects[133]. The same is true for major bleeding related to oral anticoagulant therapy[135]. These concepts are particularly important if related to the findings of our experience. As previously mentioned, in our cohort of AF individuals, three different patterns were identified at the untargeted metabolomic analysis. Accordingly, study participants could be clustered basing on the CHA_2DS_2-VASc and the SPPB scores, and to the IL-6 levels, with, in each case, the same group showing the lower results at the CHA_2DS_2-VASc and the SPPB, and the higher concentration of the cytokine. Indeed, both the Frailty Deficit Index[136] and the Phenotype Frailty Index[137], the two most important models to understand the condition, are based on the interaction among comorbidities, disability, and frailty itself (Fig. 5.1).

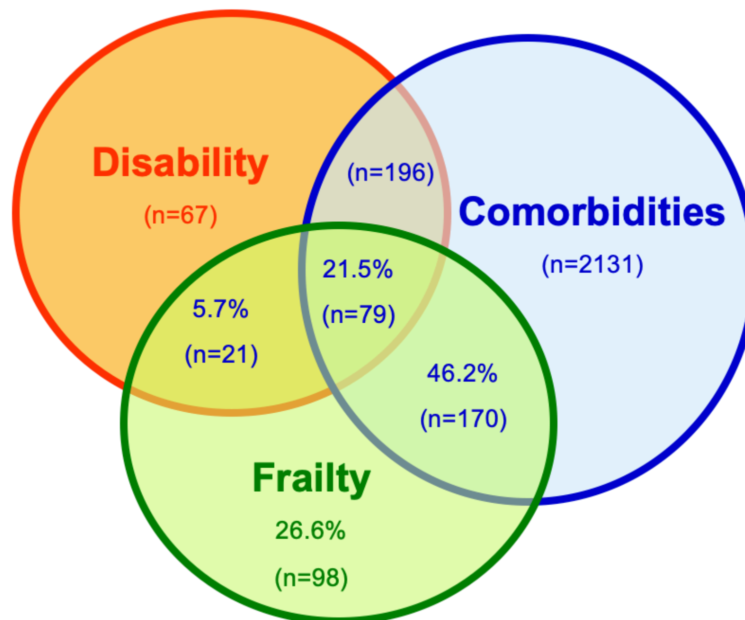


Figure 5.1: The interaction among comorbidities, disability, and frailty, as detailed in the paper by Fried et al., 2021.

We previously demonstrated that the CHA_2DS_2-VASc score, expressing a number of comorbid conditions, and for this reason an index of clinical complexity, was inversely related with cognitive function (i.e., with MMSE), and importantly with SPPB, one of the most important indexes of disability and frailty[138]. Moreover, in our main dataset, also IL-6 concentration was inversely associated with SPPB score, demonstrating once again the significant link between disability and frailty with inflamm-ageing. Indeed, because of the significant role of inflammation in cardiovascular diseases, and given that the pro-inflammatory state typical of ageing is a strong risk factor for many age-related chronic conditions, cardiovascular diseases themselves could often precede, follow, or develop in the context of multimorbidity and frailty[139]. More in general, many studies suggest that chronic inflammation is a risk factor across multiple diseases, some of which are sometimes considered unrelated. Among these are to be mentioned cardiovascular diseases, diabetes, chronic renal failure, cancer, depression, and dementia. In addition, higher levels of inflammatory markers are associated with loss of muscular tissues and strength, higher loss of mobility, lower physical performance, and depression, with all these elements necessary to define a frail condition. Accordingly, a high

concentration of IL-6 can be associated with an accelerated development of multiple chronic diseases and frailty in older individuals[139].

In our experience, IL-6 was higher also in patients with hyperuricemia. This result is consistent with those reported by other groups, showing that the cytokine levels were significantly increased in children with hyperuricemia, even if they have never had an acute gout event before[140], and in the older patients enrolled in the InChianti Study. In particular, in this study, a positive and significant correlation between uric acid levels and IL-6, and other inflammatory markers, was found in the whole enrolled population and in the subgroup of subjects who showed a normal uric acid concentration. It could be hypothesized that uric acid could be part of a complex vicious cycle involving inflammatory and oxidative-related mechanisms. Chronic inflammation causing hypoxia and cellular damage could upregulate uric acid production and trigger free radicals release. In particular, cytokines could enhance xanthine oxidase activity, the ROS-mediated cell damage and apoptosis. These alterations could subsequently originate endothelial dysfunction. The following release of uric acid in the surrounding microenvironment may further boost the inflammation cascade, even if uric acid concentration is within the normal range. Older age and the presence of comorbidities could significantly strengthen all these interactions[141].

The association between medium- long-chain acylcarnitines and IL-6 suggests an interplay between these molecules and confirms the possible involvement of acylcarnitines in the activation of proinflammatory pathways as suggested in previous studies[142]. In fact, long-chain acylcarnitines (LCAC) concentration in blood or tissues have been associated with several conditions as well as inflammation, cell stress response, and insulin resistance[143–145]. This can be explain by the fact that an increase in the concentrations of AC reflects an incomplete or impaired long-chain fatty acids (LCFA) β-oxidation. This inefficiency, also partly due to a reduced tricarboxylic acid cycle performance, leads to the accumulation of acetyl-CoA and yields chain-shortened AC that activate certain proinflammatory pathways[146]. *In vitro* studies on a skeletal muscle model showed that LCAC trigger IL-6 production and induce a rise in markers of cell permeability and death in a dose-dependent manner[144] suggesting that, under certain conditions of inefficient β-oxidation, an increase in LCAC may contribute to lipid-

associated cell stress. Furthermore, AC are known to be involved in the development of certain cardiovascular diseases[147–151]. In cardiac ischemia, an increase in tissue or blood LCAC occurs as a consequence of the inhibition of β-oxidation that causes the accumulation of hydroxy fatty acids, acyl-CoA and AC[152]. LCAC have also been associated with alterations of Ca^{2+} intracellular release[153] and are known to induce delayed afterdepolarizations and electrophysiological alterations in cardiac tissue[154]. Studies suggest that the accumulation of LCAC affects the integrity of cell membranes, which is crucial for the maintenance of homeostasis[144]. The loss of proper myocardial cell membrane function, together with derangements in ionic flux that control cardiac electrophysiology, may contribute to the development of pathologies such as arrhythmias, myopathies, and necrosis[155,156].

Arginine is a well-established marker of endothelial function and is also the precursor of nitric oxide (NO), which is produced through the activity of NO synthase[130]. Our results show significantly lower levels of Arg in patients with AF when compared to the control group without the arrhythmia. A previous study demonstrated that NO synthase expression and NO production were, respectively, 46 and 73 % lower in atrial endocardium isolated from pigs with pace-induced AF when compared to controls. Interestingly, these changes corresponded to an increased activity of the prothrombotic protein plasminogen activator inhibitor 1 (PAI-1)[157]. Additionally, subjects with embolic stroke of undetermined source showed higher values of L-arginine and a reduced carotid intima-media thickness than patients with stroke and AF[158]. Importantly, in our analysis, the relation between Arg and AF is independent and additive to that found for age. Indeed, endothelial dysfunction is highly prevalent in older subjects, and it might contribute to the development or the worsening of important age-related conditions, such as dementia, loss of physical function, hypertension, heart and renal failure[130]. Interestingly, despite the existance of conflicting evidence, it was shown that, in older individuals, Arg concentration could be increased through oral supplementation, with this change correlated to the improvement of endothelial function[130]. In our patients, we also found an inverse relation between iron levels and Arg concentration. Indeed, the association between Arg and iron is complex. NO, derived from the amino acid, exerts its action oxidizing, nitrating, and nitrosylating regulatory proteins and enzymes. Among these,

the nitrosilation of the heme iron brings to the activation of the soluble guanylyl cyclase, which catalyzes GTP conversion to cGMP, a key step in vasodilation. Historical data support the hypothesis that NO generation could be associated with cahanges in iron homeostasis, due to an enhanced iron release from intracellular ferritin stores. Also, it was shown that macrophages activated by interferon-gamma synthesize a flavoprotein promoting the conversion of Arg to NO, with this last mediato responsible of the efflux of the metal from neoplastic and infected cells. Importantly, these macrophage target cells could significantly reduce the iron uptake from plasma if the concentration of Arg is low[159]. These last findings support the existence of an inverse relationship between the concentration of iron and that of Arg or its derivative, NO. The direct association between the amino acid concetntration and the glutamic-pyruvic transaminase levels could be justified by the involvement of the enzyme in the complex catabolic cascade of Arg itself and by the key role played by the liver in its synthesis, one of the main steps of the urea cycle[160].

The possibility to support clinical behavior with laboratory data can represent a decisive point in the management of older patients with the arrhythmia. At this regard, very recently, the AF-SCREEN International Collaboration expert panel identified a series of knowledge gaps related to the care of aged individuals with AF addressed to prevent dementia, one of the most up-to-date and dreadful complications of the arrhythmia. The association between AF and cognitive decline has newly emerged and it could be explained by the reduction of cardiac output, the presence of brain silent or clinically manifest ischemic lesions, the activation of inflammation, the presence of microbleeds related to oral anticoagulant therapy, and the influence of genetic factors. Some of the issues to be clarified are related to oral anticoagulant therapy. Their aim is to understand the role of arrhythmic burden on dementia development, the need to anticoagulate patients with atrial cardiomyopathy not showing AF, and the right moment to start therapy in order to prevent cognitive decline[161] (Fig. 5.2).

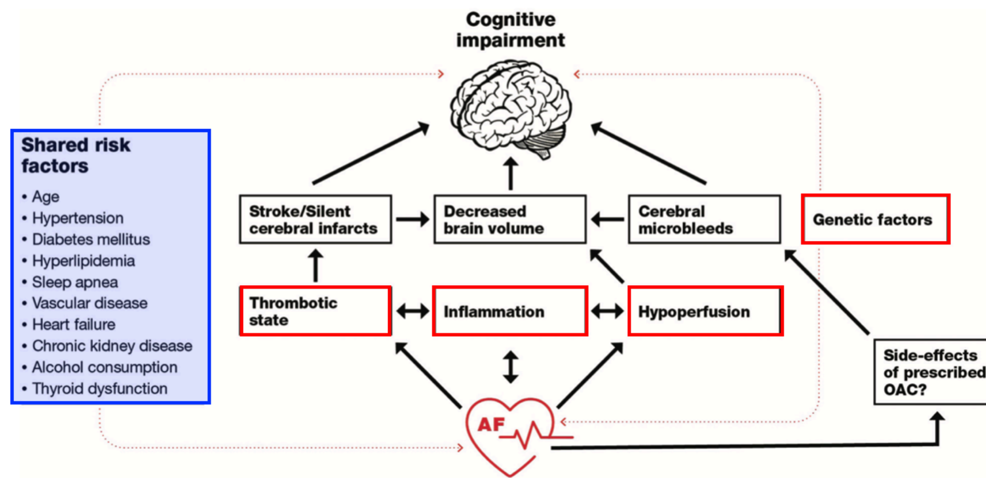


Figure 5.2: The suspected mechanisms of cognitive impairment in atrial fibrillation. From Rivard et al., 2022.

The possibility to compare the metabolic pathways and the concentration of inflammatory mediators in patients with AF and in controls, should allow to identify some important factors potentially useful to guide clinical practice. Indeed, even if in a completely preliminary and exploratory analysis, our findings allowed to demonstrate the reduced concentration of Arg - a condition potentially indicating endothelial dysfunction - in subjects with the arrhythmia. These results could allow to better describe atrial cardiomyopathy, and could justify the increased risk of thromboembolic events in patients with a history of AF even in the absence of the arrhythmia itself. It is not clear whether the identification of biomarkers could, in the long term, replace the clinical diagnosis of comorbidities and frailty, but metabolomics studies can certainly enhance a more objective and quantifiable assessment of the pathophysiological alterations linked to AF and its comorbidities including frailty[162]. Furthermore, a metabolomic approach could help to identify the characteristics of subjects with silent AF, as well as of those at risk of developing the arrhythmia, especially the frail ones. In this perspective, metabolomic analysis could reveal an useful preventive tool. The other important issues raised by the experts of the AF-SCREEN International Collaboration were about the choice between the rate- or the rhythm-control strategy of the arrhythmia. New evidence on this topic originated by the researchers involved in the Early Treatment of Atrial Fibrillation for

Stroke Prevention Trial (EAST-AFNET 4), who demonstrated that an early intervention ($<$ 1 y from the diagnosis of AF) to restore and maintain sinus rhythm, when compared to usual care, was correlated to the reduced incidence of the composite outcome, consisting of cardiovascular death, stroke, and new hospitalizations due to heart failure or acute coronary syndromes[163] (Fig. 5.3).

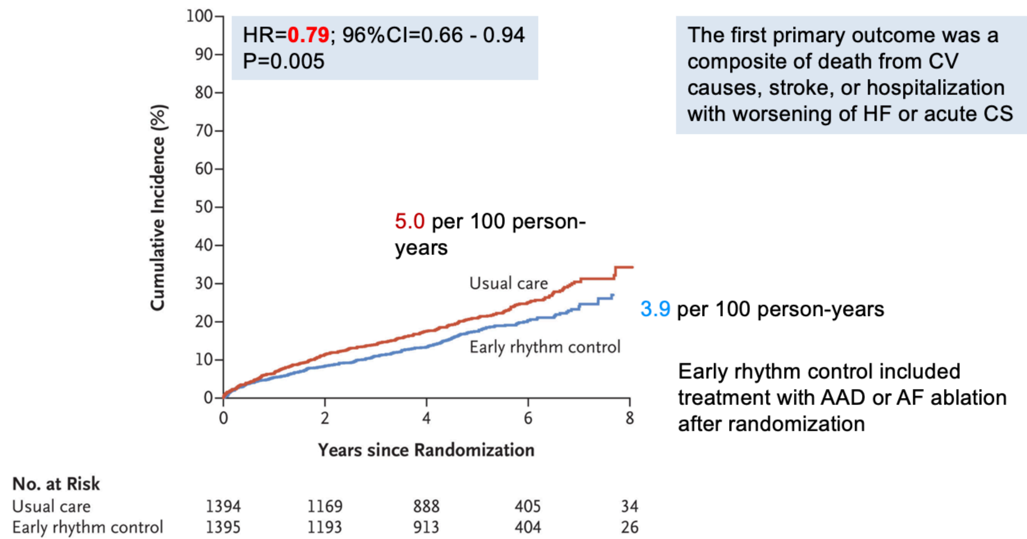
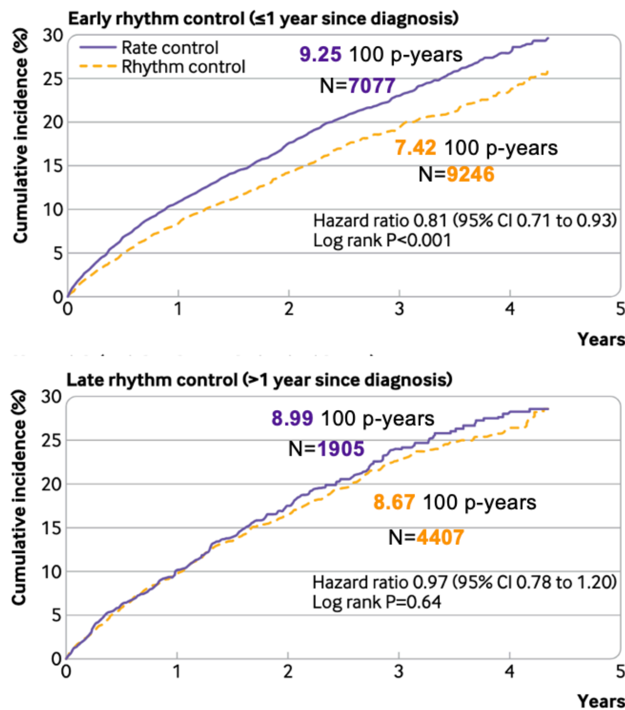


Figure 5.3: Cumulative-incidence curves for the first primary outcome (early rhythm control – N = 1,395, 70 y; usual care – N = 1,394, 70 y; follow-up: 5.1 y). From Kirchhof et al., 2020.

The same results on identical endpoints were obtained in a huge registry, in which the rhythm control strategy resulted once again superior to a rate-control one if started in the first 12 months from the arrhythmia onset. No benefit was found in subjects treated after that period[164] (Fig. 5.4).



Outcomes
The primary outcome was a composite of: death from CV causes, ischaemic stroke, admission to hospital due to HF, or AMI

Figure 5.4: Incidence of the primary outcome in early and late treatments for AF among the NHIS participants (early/late - N = 16,323/6,312; age: 70/69 years; CHA2DS2-VASc: 4/4; FU: 2.1/2.2 years; 2005-2015). From Kim et al., 2021.

Even the substrate ablation of AF revealed to be a more effective strategy to reduce the incidence of dementia than medical therapy. Importantly, the clinical benefits were observed in both vascular and Alzheimer's dementia[165]. It was also found that a rhythm-control strategy to be effective on cognitive decline should be pursued in patients with a maximum age of about 85 years[166] .

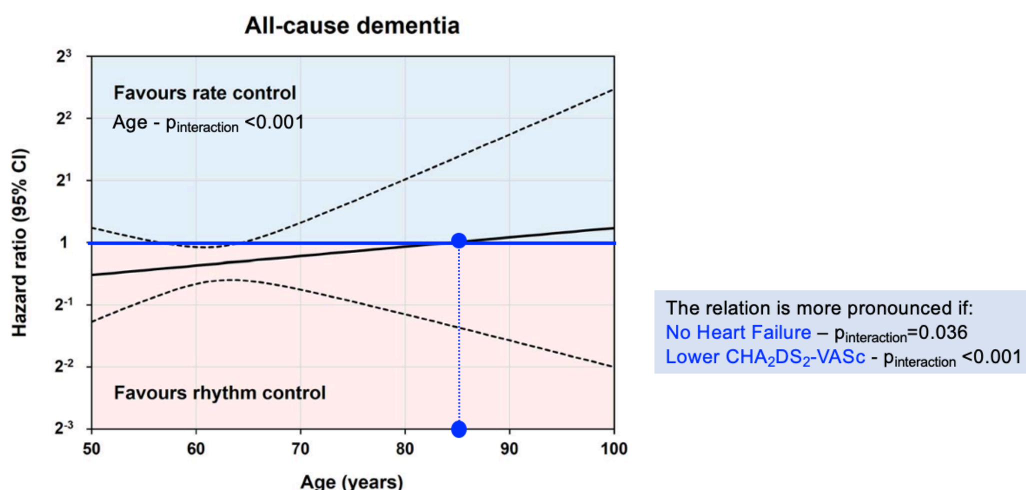


Figure 5.5: Relation between age at treatment initiation and risk of dementia for rhythm-control or rate-control among the NHIS participants. From Kim et al., 2022.

Accordingly, the knowledge gaps about the arrhythmia and dementia concern the effects of AF ablation, the role of anti-arrhythmic therapy, irregularity of rhythm, and of a rate-control strategy[161]. We think that to be able to successfully choose the more appropriate type of therapy - sometimes a highly-invasive one - the support of the biochemical basis of a clinical “scenario” could be extremely useful.

This study was conducted on a small sample of patients and controls and this could represent a limitation. Additionally, untargeted metabolomics produces a global biochemical phenotype and the challenges of a comprehensive investigation of the metabolome are well known. In fact, beside the diversity in physical and chemical properties of the metaboolites, they also occur in a wide concentration range. Furthermore, the dynamics of the metabolome should also be considered as metabolite distribution can vary according to several factors including diet or circadian fluctuations[167]. Obtaining more information about the diet and lifestyle of the subjects enrolled in the study could have been useful to better stratify the subjects in more homogenous groups, thus minimizing the differences that are not strictly due to the presence or absence of AF. In spite of these limitations, the differences we found allowed to build statistical models biologically and clinically plausible. It should be mentioned that this experience derived from the activity of only one center. This fact could limit the generalizability of our conclusions

but, also, it should make more homogeneous the experimental conduct of the team. We included only patients with persistent or long-standing persistent forms of the arrhythmia. We cannot exclude that our conclusions could have been different if we had studied also patients with paroxysmal (short-lasting episodes) or permanent AF. However, a persistent form of the arrhythmia is very frequently observed in older people. All patients enrolled in our study presented a form of symptomatic AF and therefore we could not evaluate the metabolomic profile of subjects with silent AF. Also, we could not exclude that any of the healthy elderly subjects presented a silent form of AF, although it is highly unlikely since they did not have any of the risk factors or comorbidities associated with the arrhythmia. Lastly, for statistical reasons, the vast number of molecules that were detected did not allow to test all possible interactions. However, this study is to be conceived as a true pilot one. From the robust protocol we developed and the findings we presented, we think it could pave the way to more up-to-date and refined projects.

In conclusion, the results of this study allowed to reach some conclusions that we feel important for a more effective clinical practice. First, healthy young subjects differed from AF patients because of their body size, probably a surrogate marker of sarcopenia. Second, applying metabolomics, AF patients could be clustered according to their clinical complexity, their physical function and the plasma levels of IL-6. Accordingly, the subjects with the arrhythmia presenting the worse metabolic profile could represent the frailest ones. A more "targeted" analysis could allow to identify some of the metabolic markers associated with the condition and to decide if an intervention is appropriate or futile. Third, we found an association between inflammation and medium-long-chain acylcarnitines which may cause electrophysiological alterations in cardiac tissue and thus contribute to the establishment of a favourable substrate for AF development. Last, we identified the existence of endothelial dysfunction in our cohort of patients. In them, AF acted independently and on top of the age-related effects to contribute to atrial cardiomyopathy and to an increased risk of stroke. As previously mentioned, more specific projects with a more adequate sample size are needed to confirm our findings and future studies should follow the metabolomic profile changes

that occur as AF progresses.

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Appendix

Table 6.1: The metadata available of patients with AF*.

Biometric data	Heart details	Comorbidities	Drugs	GMA & other scores	Blood test values
Age	RCAVI	hypertension	type of oral anticoagulant	MMSE	leucocites
Height	LCAVI	diabetes	α blockers	GDS	hemoglobin
Weight	RABI	dyslipidemia	β blockers	SPPB - total	platelets
BMI	LABI	CKD	ACE inhibitors/ARBs	CHA_2DS_2-VASc score	ESR
Gender	left atrial diameter	hyperuricemia	dihydropyridines		fibrinogen
	left atrial area	CAD	statins		glycemia
	left atrial volume	CHF	diuretics		creatinine
	EDD	CVD	amiodarone		Na^+
	ESD	arteriopathy	antiaggregants		K^+
	EF	thyroid disease	antiulcer drugs		Cl^-
		COPD	antidiabetic drugs		Ca^{2+}
			insulin		ALT/GPT
			anti uric acid drugs		triglycerides
			thyroxine		cholesterol
			benzodiazepines		HDL
			SSRIs/SNRIs		uricemia

Biometric data	Heart details	Comorbidities	Drugs	GMA & other scores	Blood test values
			digitalis		CG eGFR ferritin iron NT-proBNP CRP

*Biometric data, habits, comorbidities, and drugs are also available for the control group.

Table 6.2: Frequency table of categorical variables.

Variable	N yes	% yes
smoke	26	52
hypertension	43	86
diabetes	8	16
dyslipidemia	22	44
CKD	10	20
hyperuricemia	15	30
CAD	13	26
CHF	17	34
CVD	6	12
arteriopathy	10	20
thyroid disease	10	20
COPD	9	18
α blockers	12	24
β blockers	39	78
ACE inhibitors/ARBs	42	84

Variable	**N yes**	**% yes**
dihydropyridines	10	20
statins	23	46
diuretics	32	64
amiodarone	23	46
antiaggregants	6	12
antiulcer drugs	23	46
antidiabetic drugs	5	10
insulin	3	6
anti uric acid drugs	10	20
thyroxine	6	12
benzodiazepines	5	10
SSRIs/SNRIs	3	6
digitalis	18	36
lives alone	11	22

Table 6.3: The clusters obtained with k-means clustering of metabolomics GC-MS data of H and AF.

Sample	Cluster 1	Cluster 2
340_AF		X
341_AF		X
361_AFHF	X	
358_AF	X	
356_AF		X
355_AFHF		X
354_AFHF		X
351_AF	X	
353_AF	X	
348_AFHF	X	

Sample	Cluster 1	Cluster 2
346_AF	X	
343_AFHF		X
342_AF		X
362_AFHF	X	
363_AF		X
349_AFHF	X	
347_AF	X	
365_AFHF		X
366_AFHF		X
369_AFHF		X
370_AFHF		X
371_AF		X
374_AF	X	
376_AFHF		X
380_AFHF		X
381_AFHF		X
387_AFHF		X
389_AFHF		X
390_AFHF	X	
391_AFHF	X	
393_AFHF		X
394_AF		X
396_AFHF		X
398_AFHF	X	
397_AF	X	
405_AF		X
406_AF	X	
407_AFHF		X
408_AFHF		X

Sample	Cluster 1	Cluster 2
409_AF		X
410_AF		X
455_AF		X
465_AFHF		X
466_AFHF		X
454_AF	X	
3_Hy	X	
4_Hy	X	
5_Hy		X
7_Hy		X
9_Hy		X
10_Hy		X
11_Hy		X
12_Hy		X
50_Ho	X	
51_Ho	X	
52_Ho		X
53_Ho		X
54_Ho		X
55_Ho	X	
56_Ho		X
57_Ho	X	
58_Ho		X
59_Ho	X	
60_Ho		X
61_Ho		X
63_Ho		X

Table 6.4: The clusters obtained with k-means clustering of metabolomics GC-MS data of AF_{only} and AF_{HF}.

Sample	Cluster 1	Cluster 2
340_AF		X
341_AF		X
361_AFHF	X	
358_AF	X	
356_AF		X
355_AFHF		X
354_AFHF		X
351_AF	X	
353_AF	X	
348_AFHF	X	
346_AF	X	
343_AFHF		X
342_AF		X
362_AFHF	X	
363_AF		X
349_AFHF	X	
347_AF	X	
365_AFHF		X
366_AFHF		X
369_AFHF		X
370_AFHF		X
371_AF		X
374_AF	X	
376_AFHF		X
380_AFHF		X
381_AFHF		X
387_AFHF		X

Sample	Cluster 1	Cluster 2
389_AFHF		X
390_AFHF	X	
392_AFHF	X	
393_AFHF		X
394_AF		X
396_AFHF		X
398_AFHF	X	
397_AF	X	
405_AF		X
406_AF	X	
407_AFHF		X
408_AFHF		X
409_AF		X
410_AF		X
455_AF		X
465_AFHF		X
466_AFHF		X
454_AF	X	