Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN

Farmacologia e Oncologia Molecolare

Ciclo XVIII

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Association between amount of injured heart and Novel molecular/bio markers in myocardial infarction

Settore Scientifico Disciplinare BIO/12

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1. INTRODUCTION

1.1 CARDIOVASCULAR DISEASES: AN OVERVIEW OF THE PROBLEM

Cardiovascular diseases (CVD) comprise the most prevalent serious disorders in the developed nations. The American Heart Association has reported that in 2002, 62 million Americans—32 million females and 30 million males (i.e., more than one in five persons)—had a cardiovascular disease (including hypertension). The prevalence rises progressively with age from 5% at age 20 to 75% at age75 years. Americans suffer from angina pectoris and more than 1 million experience a myocardial infarction each year. About 4.8 million Americans have congestive heart failure and more than half a million new cases occur each year. Hospitalizations for heart failure have risen from 400,000 to 950,000 per year in the past 20 years. More than 1.4 million patients undergo cardiac catheterization each year, and approximately 1.2 million undergo revascularization (either percutaneous coronary intervention or coronary artery bypass grafting).

Among developed nations, death rates from cardiovascular diseases are highest in the nations of the former Soviet Union, are intermediate in the United States and Western Europe, and lowest in Japan. The prevalence of cardiovascular disease, especially coronary artery disease, is rising alarmingly in China, India, Pakistan, and the Middle East, as nutritional and infectious causes of death decline in these regions. It has been projected that by 2020 cardiovascular diseases will be the leading causes of death worldwide.

In particolar, in Europe, each year CVD causes over 4 million deaths and over 1.9 million deaths in the European Union (EU). Nearly half (47%) of all deaths are from CVD (52% of deaths in women and 42% of deaths in men). The main forms of CVD are coronary heart disease (CHD) and stroke.

CVD is the main cause of death in women in all countries of Europe. CVD mortality is now falling in most European countries, including Central and Eastern European countries which saw large increases until the beginning of the 21st century. Overall CVD is estimated to cost the EU economy almost €196 billion a year. Of the total cost of CVD in the EU, around 54% is due to health care costs, 24% due to productivity losses and 22% due to the informal care of people with CVD.

1.2 Definition of myocardial infarction

The arrival of sensitive and specific serologic biomarkers has led to a change in the definitions of myocardial infarction. The World Health Organisation had defined myocardial infarction as a combination of two of three characteristics: typical symptoms (i.e., chest pain/discomfort), enzymes rise and a typical electrocardiogram pattern involving the development of Q waves (1). The arrival of biomarkers has led to the introduction of the term 'acute coronary syndrome' which now includes all those with ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI) and unstable angina.

When patients with acute ischemic discomfort are first seen, the working diagnosis is that they are suffering from an acute coronary syndrome. The 12-lead electrocardiogram (ECG) is at the center of the decision pathway for management since it permits distinction of those patients presenting with ST-segment elevation from those presenting without ST-segment elevation. Serum cardiac biomarkers are obtained to distinguish unstable angina from non-STsegment MI (NSTEMI) and to assess the magnitude of an ST-segment elevation MI (STEMI).

1.3 Prevalence of myocardial infarction

Acute myocardial infarction (AMI) is one of the most common diagnoses in hospitalized patients in industrialized countries. In the United States, approximately 650,000 patients experience a new AMI and 450,000 experience a recurrent AMI each year. The early (30-day) mortality rate from AMI is 30%, with more than half of these deaths occurring before the stricken individual reaches the hospital. Although the mortality rate after admission for AMI has declined by 30% over the past two decades, approximately 1 of every 25 patients who survives the initial hospitalization dies in the first year after AMI. Survival is markedly reduced in elderly patients (over age 75).

Furthermore, from a predominance of STEMI in the past, STEMIs are declining in prevalence (2), with the overall proportion of NSTEMI increasing (3). The multinational Global Registry of Acute Coronary Events (GRACE) project reported data from its first 11543 patients in 2002 (4). Of these patients included, 38% had a final diagnosis of unstable angina, 30% had STEMI, and 25% had NSTEMI. The ENACT study in 1999 on

patients hospitalised with acute coronary syndromes in 390 hospitals in 29 European countries reported that unstable angina was the most frequent cause of hospitalisation (46%), followed by acute myocardial infarction (39%) (5). In a prospective survey of the characteristics, treatments and outcomes of patients with acute coronary syndromes in Europe, the Euro Heart Survey of Acute Coronary Syndromes reported an initial diagnosis of STEMI in 42.3%, NSTEMI in 51.2%, and 'undetermined electrocardiogram acute coronary syndrome' in 6.5% (6, 7).

1.4 Risk factors

Six primary risk factors have been identified with the development of atherosclerotic coronary artery disease and MI: hyperlipidemia, diabetes mellitus, hypertension, tobacco use, male gender, and family history of atherosclerotic arterial disease. The presence of any risk factor is associated with doubling the relative risk of developing atherosclerotic coronary artery disease (8).

In White populations, aetiologic risk factors for myocardial infarction such as hypertension, diabetes and smoking are in addition prognostic factors (9, 10). In patients diagnosed with coronary heart disease who smoked, a systematic review of cohort studies showed a 36% reduction in crude relative risk of mortality for patients who quit compared with those who continued smoking (11). Furthermore, South Asian patients, from Bangladeshis in London to Indians in the USA, present with coronary disease at younger ages than White patients (12, 13), age being the most important non-modifiable risk factor in the aetiology and prognosis of coronary disease from large population studies such as Framingham (14) and the World Health Organisation MONICA Project (multinational monitoring of trends and determinants in cardiovascular disease) (15).

1.5 Treatment of myocardial infarction

Treatment of patients with an acute coronary syndrome who present with STsegment elevation on the ECG (STEMIs) is principally aimed at restoration of myocardial perfusion (reperfusion) using fibrinolysis (thrombolytic drugs) or increasingly primary percutaneous coronary intervention (PCI, angioplasty/stenting). However, NSTEMIs and unstable angina are more heterogeneous in their presentation and are associated with a higher variation in diagnosis and treatment (16). Variations in receipt of medical treatment such as statins as well as access to angiography and subsequent receipt of coronary revascularisation are widely present in such patients (17). The wide range of clinical 41 manifestations and hence treatments will result in variable prognoses. Reasons behind these variations may include: - the use of different definitions for 'unstable angina' and 'NSTEMI'(16) - differences in the characteristics of presenting patients - geographical practice variation (18) which is influenced by the incidence of coronary heart disease in the local population, the type of resources available, and doctors' perceptions of existing therapies. It is conceivable that ethnic differences will exist in treatment and hence prognosis and explaining reasons behind such differences will entail consideration of not only biological differences but also socio-demographic and clinical management variables.

Markers of myocardial injury are often obtained in the emergency department evaluation of acute chest discomfort. The most commonly used markers are creatine kinase (CK), CK-MB, and the cardiac troponins (I and T). Rapid bedside assays of the cardiac troponins have been developed and shown to be sufficiently accurate to predict prognosis and guide management. Some data support the use of other markers, such as serum myoglobin, C-reactive protein (CRP), and B-type natriuretic peptide (BNP); their roles are the subject of ongoing research. Single values of any of these markers do not have high sensitivity for acute myocardial infarction or for prediction of complications. Hence, decisions to discharge patients home should not be made on the basis of single negative values of these tests.

1.6 Pathophysiology: role of acute plaque rupture

STEMI generally occurs when coronary blood flow decreases abruptly after a thrombotic occlusion of a coronary artery previously affected by atherosclerosis. Slowly developing, high-grade coronary artery stenoses do not usually precipitate STEMI because of the development of a rich collateral network over time. Instead, STEMI occurs when a coronary artery thrombus develops rapidly at a site of vascular injury. This injury is produced or facilitated by factors such as cigarette smoking, hypertension, and lipid accumulation. In most cases, infarction occurs when an atherosclerotic plaque fissures, ruptures, or ulcerates and when conditions (local or systemic) favor thrombogenesis, so that a muralthrombus forms at the site of rupture and leads to coronary artery occlusion. Histologic studies indicate that the coronary claque prone to rupture are those with a rich lipid core and a thin fibrous cap. After an initial platelet monolayer forms at the site of the ruptured plaque, various agonists (collagen, ADP, epinephrine, serotonin) promote platelet activation. After agonist stimulation of platelets, there are production and release of thromboxane A2 (a potent local vasoconstrictor), further platelet activation, and potential resistance to thrombolysis. In addition to the generation of thromboxane A 2, activation of platelets by agonists promotes a conformational change in the glycoprotein IIb/IIIa receptor. Once converted to its functional state, this receptor develops a high affinity for amino acid sequenze on soluble adhesive proteins (i.e., integrins) such as von Willebrand factor (vWF) and fibrinogen. Since vWF and fibrinogen are multivalent molecules, they can bind to two different platelets simultaneously, resulting in platelet cross-linking and aggregation.

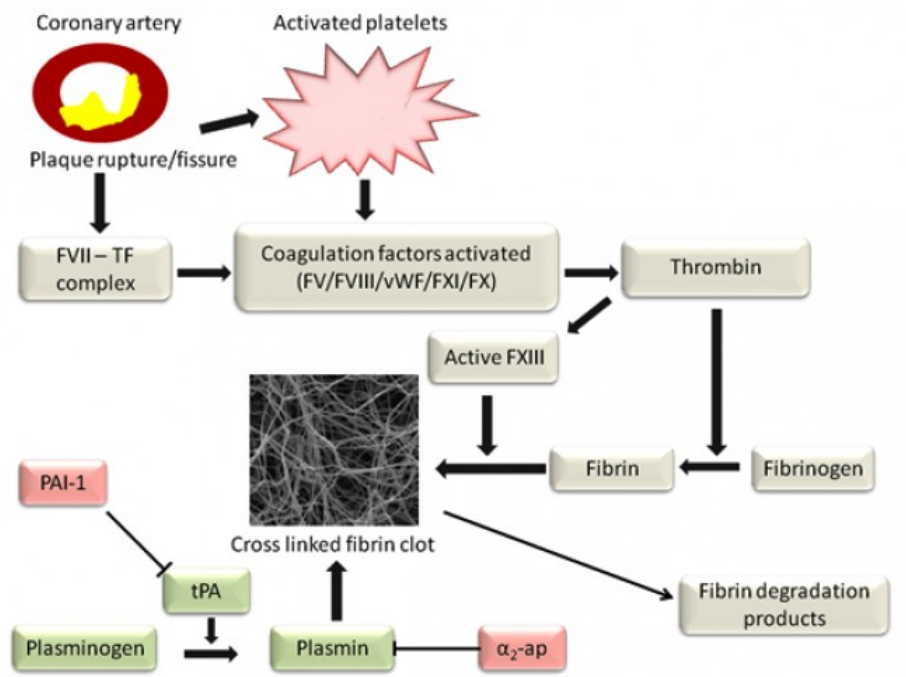

Figure 1. Mechanism of plaque rupture. *Simplified illustration of fibrin clot formation and fibrinolysis. Following plaque rupture or fissure, a TF/FVII complex is formed and platelets are activated. This results in the activation of numerous coagulation factors and ultimately the conversion of prothrombin to thrombin, which converts soluble fibrinogen to fibrin. A network of fibrin fibres is formed which is stabilised by the actions of FXIII, cross linking the fibres. Clot lysis occurs following the conversion of plasminogen to plasmin by tissue plasminogen activator (tPA) which generates fibrin degradation products. Fibrinolysis is regulated by plasminogen activator inhibitor-1(PAI-1) which inhibits tPA and α2-antiplasmin (α2-ap) which inhibits plasmin activity.*

1.7 Coagulation cascade

The coagulation cascade is activated on exposure of tissue factor in damaged endothelial cells at the site of the ruptured plaque. Factors VII and X are activated, ultimately leading to the conversion of prothrombin to thrombin, which then converts fibrinogen to fibrin. Fluid-phase and clot-bound thrombin participate in an autoamplification reaction that leads to further activation of the coagulation cascade. The culprit coronary artery eventually becomes occluded by a thrombus containing platelet aggregates and fibrin strands. In rare cases, STEMI may be due to coronary artery occlusion caused by coronary emboli, congenital abnormalities, coronary spasm, and a wide variety of systemic—particularly inflammatory—diseases. The amount of myocardial damage caused by coronary occlusion depends on (1) the territory supplied by the affected vessel, (2) whether or not the vessel becomes totally occluded, (3) the duration of coronary occlusion, (4) the quantity of blood supplied by collateral vessels to the affected tissue, (5) the demand for oxygen of the myocardium whose blood supply has been suddenly limited, (6) native factors that can

produce early spontaneous lysis of the occlusive thrombus, and (7) the adequacy of myocardialperfusion in the infarct zone when flow is restored in the occluded epicardial coronary artery. Patients at increased risk of developing STEMI include those with multiple coronary risk factors and those with unstable angina or Prinzmetal's variant angina. Less common underlying medical conditions predisposing patients to STEMI include hypercoagulability, collagen vascular disease, cocaine abuse, and intracardiac thrombi or masses that can produce coronary emboli.

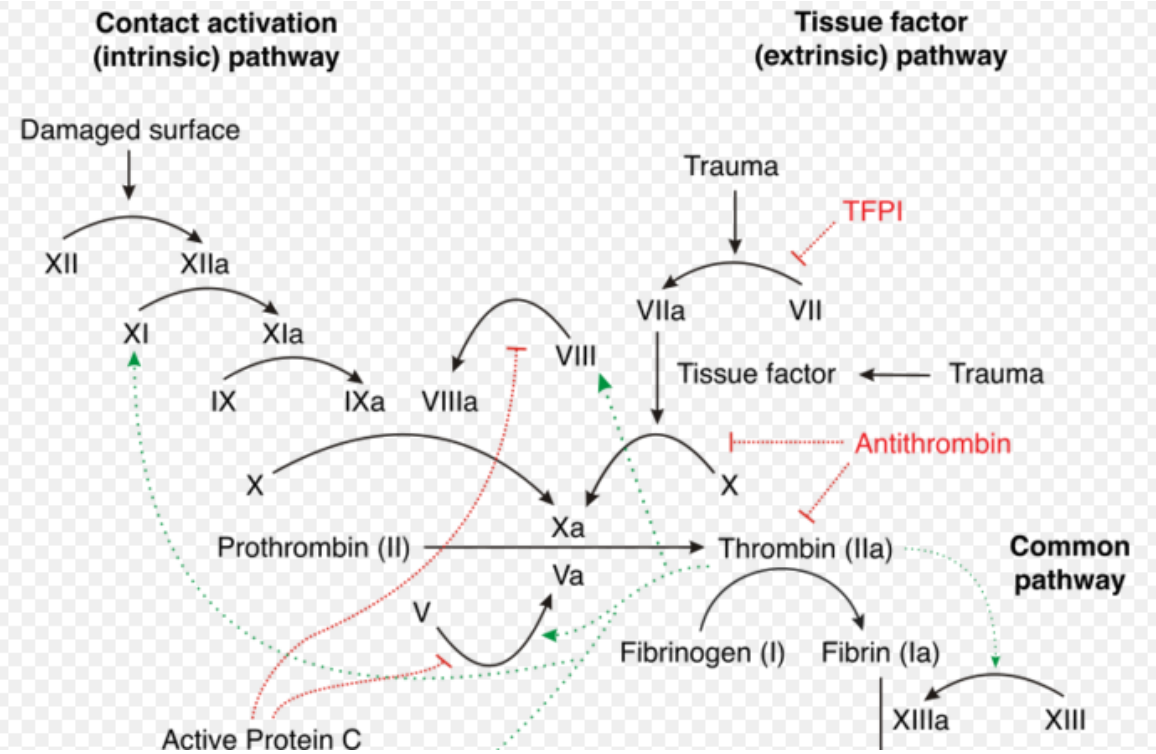

Figure 2. Coagulation cascade. *Coagulation with arrows for negative and positive feedback.*

1.8 Laboratory findings

Myocardial infarction (MI) progresses through the following temporal stages: (1) acute (first few hours to 7 days), (2) healing (7 to 28 days), and (3) healed (29 days). When evaluating the results of diagnostic tests for STEMI, the temporal phase of the infarction process must be considered. The laboratory tests of value in confirming the diagnosis may be divided into four groups: (1) ECG, (2) serum cardiac biomarkers, (3) cardiac imaging, and (4) nonspecific indexes of tissue necrosis and inflammation.

1.8.1 Electrocardiogram

During the initial stage of the acute phase of MI, total occlusion of an epicardial artery produces STsegment elevation. Most patients initially presenting with ST-segment elevation evolve Q waves on the ECG and are ultimately diagnosed as having sustained a Q-wave MI. A small proportion may sustain only a non-Q-wave MI. When the obstructing thrombus is not totally occlusive, obstruction is transient, or if a rich collateral network is present, no ST-segment elevation is seen. Such patients are initially considered to be experiencing either unstable angina or NSTEMI. Among patients presentino without ST-segment elevation, if a serum cardiac biomarker of necrosis is detected and no Q wave develops, the diagnosis of non-Q-wave MI is ultimately made. A minority of patients who present initially without ST-segment elevation may develop a Q-wave MI. Previously it was believed that transmural MI is present if the ECG demonstrates Q waves or loss of R waves, and nontransmural MI may be present if the ECG shows only transient ST-segment and T-wave changes. However, electrocardiographic-pathologic correlations are far from perfect; therefore a more rational nomenclature for designating electrocardiographic infarction is now commonly in use, with the terms Q-wave MIandnon-Q-wave MIreplacing the termstransmural MIandnontransmural MI, respectively.

1.8.2 Serum cardiac biomarkers

Certain proteins, called serum cardiac markers, are released into the blood in large quantities from necrotic heart muscle after STEMI. The rate of liberation of specific proteins differs depending on their intracellular location and molecular weight and the local blood and lymphatic flow. The temporal pattern of protein release is of diagnostic importance, but contemporary urgent reperfusion strategies necessitate making a decision (based largely on a combination of clinical and ECG findings) before the results of blood tests have returned from the central laboratory. Rapid whole-blood bedside assays for serum cardiac markers are now available and may facilitate management decisions, particularly in patients with nondiagnostic ECGs. Creatine phosphokinase (CK) rises within 4 to 8 h and generally returns to normal by 48 to 72 h. An important drawback of total CK measurement is its lack of specificity for STEMI, as CK may be elevated with skeletal muscle trauma. A two - to threefold elevation of total CK may follow an intramuscular injection, for example. This ambiguity may lead to the erroneous diagnosis

of STEMI in a patient who has been given an intramuscular injection of a narcotic for chest pain of noncardiac origin. Other potential sources of total CK elevation are (1) skeletal muscular diseases, including muscular dystrophy, myopathies, and polymyositis; (2) electrical cardioversion; (3) hypothyroidism; (4) stroke; (5) surgery; and (6) skeletal muscle damage secondary to trauma, convulsions, and prolonged immobilization. The MB isoenzyme of CK has the advantage over total CK that it is not present in significant concentrations in extracardiac tissue and therefore is considerably more specific. However, cardiac surgery, myocarditis, and electrical cardioversion often result in elevated serum levels of the MB isoenzyme. A ratio (relative index) of CKMB mass: CK activity suggests but is not diagnostic of a myocardial rather than a skeletal muscle source for the CKMB elevation. This ratio is less useful when levels of total CK are high owing to skeletal muscle injury or when the total CK level is within the normal range but CKMB is elevated. Cardiac-specific troponin T(cTnT) and cardiac-specific troponin I(cTnI) have amino acid sequences different from those of the skeletal muscle forms of these proteins. These differences permitted the development of quantitative assays for cTnT and cTnI with highly specific monoclonal antibodies. Since cTnT and cTnI are not normally detectable in the blood of healthy individuals but may increase after STEMI to levels 20 times higher than the upper reference limit, the noise level of the assay, the measurement of cTnT or cTnI is of considerable diagnostic usefulness, and they are now the preferred biochemical markers for MI. The cardiac troponins are particularly valuable when there is clinical suspicion of either skeletal muscle injury or a small MI that may be below the detection limit for CK and CKMB measurements. Levels of cTnI and cTnT may remain elevated for 7 to 10 days after STEMI. Myoglobinis released into the blood within only a few hours of the onset of STEMI. Although myoglobin is one of the first serum cardiac markers that rises above the normal range after STEMI, it lacks cardiac specificity, and it is rapidly excreted in the urine, so that blood levels return to the normal range within 24 h of the onset of infarction. Many hospitals are using cTnT or cTnI rather than CKMB as the routine serum cardiac marker for diagnosis of STEMI, although any of these analytes remains clinically acceptable. It is not cost-effective to measure both a cardiac-specific troponin and CKMB at all time points in every patient. However, in view of the prolonged elevation of cardiac-specific troponins (1 week), episodes of recurrent ischemic discomfort and suspected recurrent MI are more readily diagnosed with a serum cardiac marker that remains elevated in the blood more briefly, such as CKMB or myoglobin. While it has long been recognized that the total quantity of protein released correlates with the size of

the infarct, the peak protein concentration correlates only weakly with infarct size. Recanalization of a coronary artery occlusion (either spontaneously or by mechanical or pharmacologic means) in the early hours of STEMI causes earlier and higher peaking (at about 8 to 12 h after reperfusion) of serum cardiac markers. For the purposes of confirming the diagnosis of MI, serum cardiac markers should be measured on admission, 6 to 9 h after admission, and 12 to 24 h after admission if the diagnosis remains uncertain. The non specific reactionto myocardial injury is associated with polymorphonuclear leukocytosis, which appears within a few hours after the onset of pain and persists for 3 to 7 days; the white blood cell count often reaches levels of 12,000 to 15,000/L. The erythrocyte sedimentation rate rises more slowly than the white blood cell count, peaking during the first week and sometimes remaining elevated for 1 or 2 we.

1.9. Heart Failure: definition

Congestive heart failure (CHF), also known as heart failure (HF) is an individual and societal challenge. CHF is a chronic cardiovascular syndrome characterized by the inability of the heart to fill with or eject blood due to any structural or functional cardiac conditions (19) and in which patients have symptoms of shortness of breath and/or fatigue, show signs of fluid retention and have an abnormality of the heart at rest (20). The Canadian Heart and Stroke Foundation (CHSF) (2009) defines HF as a condition that develops after the heart has been damaged or weakened in relation to medical events such as: 1) coronary artery disease (CAD), a narrowing of the arteries that supply blood to the heart; 2) past myocardial infarction (MI), a previous heart attack that has left scar tissue in the heart that impedes function; 3) hypertension (HTN), high blood pressure; 4) cardiac valve disease, from rheumatic fever or other illnesses; 5) cardiomyopathy, a primary disease of the cardiac muscle itself; 6) congenital heart defects, defects that have occurred from birth, and; 7) endocarditis 11 and/or myocarditis, infection of the heart valves or heart muscle (American Heart Association (AHA)). Due to the nature of its presentation and progression, HF reduces quality of life, exercise tolerance and survival (21).

1.10. Prevalence and Incidence

CHF is a progressive disorder threatening daily functioning and resulting in reduced life expectancy (22, 23). Overall, 40% of CHF patients admitted to the hospital are readmitted or even die within one year. Overall, 50% of CHF patients die within four years (22). The prevalence of CHF is estimated at 1-3% in the western world, with incidences approaching 5-10 per 1000 people per year (22, 24). Age is an important factor affecting these numbers. In persons older than 50 years the prevalence and incidence of CHF increase progressively (22, 24). The mean age of CHF patients in the western world is 75 years, and the prevalence of CHF in 70 to 80-year-olds reaches 10-20% (22, 24). The CHF burden is expected to increase substantially over at least the next two decades (25). A steady increase in the number of CHF patients and hospital admissions for CHF is predicted because of the ageing population, success in prolonging survival in patients suffering coronary events, and success in postponing coronary events by effective primary and secondary prevention (22). These changes in the age structure of the population and improved treatment options will further fuel the CHF epidemic in western societies and impose an enormous burden on society in terms of CHF-associated healthcare costs (22). In Europe, CHF already accounts for about 2% of National expenditure on healthcare, mostly due to the cost of hospital admissions (26). CHF substantially affects patients' quality of life as it impacts daily life in terms of physical, social and emotional functioning (27). Several studies indicate that quality of life may be even more severely impaired by CHF than by other highly prevalent chronic diseases such as chronic obstructive pulmonary disease (COPD), arthritis, or angina (28, 29). Regarding physical functioning, the exercise capacity of CHF patients is often reduced due to cardiac dysfunction and symptoms of breathlessness and fatigue (30, 31). Consequently, patients become physically impaired and lose independence as daily activities can no longer be performed to the same level as before CHF diagnosis (32, 33). In addition, patients can no longer fulfil the demands of their former social life as these physical limitations hinder the accomplishment of personal and professional roles.

1.11. Heart failure characteristics

HF typically presents as episodes of acute exacerbation, interspersed with periods of clinical stability. Symptoms of HF generally include a combination of: difficulty 12

breathing (including orthopnea and dyspnea), exercise intolerance (including fatigue and weakness), dependent edema, cough, weight gain, abdominal distension, nocturia, and cool extremities (21). Less common symptoms can also include: oliguria, abdominal discomfort, nausea, anorexia, cyanosis, cognitive impairment or delirium. Symptoms of HF vary person-to-person, depending on factors such as treatment adherence and the individual's capacity to compensate for inadequate cardiac function. Heart failure is considered a progressive disease that is perpetuated by progressive left ventricular (LV) dilation and loss of cardiac contractility (34). In the past 20 years, due to the further understanding of the pathophysiological mechanisms of HF, and improvement in treatment and management strategies, the prognosis of HF, although still poor, has improved. Many patients today experience longer periods of stability between exacerbations of their HF, experience less symptom burden and have improved cardiac function (21). Despite these advancements in therapeutics, the prognosis of HF remains poor, and mortality and morbidity rates remain high. As such, there continues to be a need for further development in treatment and management strategies.

2. FACTOR XIII

COAGULATION FACTOR XIII has a transglutaminase activity that forms γ glutamyl ε lysine isopeptide bonds between fibrin monomers (35). This cross-linking confers added strength to the clot and increases its resistance to degradation by plasmin FXIII is essential for maintaining hemostasis by stabilizing the fibrin clot and protecting it from fibrinolytic degradation (36). Its deficiency results in severe bleeding diathesis and patients with FXIII deficiency usually need life-long substitution therapy (37). Inherited deficiencies of factor XIII lead to delayed bleeding after trauma and poor wound healing. Recently, FXIII has also been implicated in the risk of atherothrombotic diseases and venous thromboembolism (38). There are two forms of FXIII, the one that exists in the plasma (pFXIII) is a tetramer consisting of two potentially active FXIII-A and two inhibitory/carrier FXIII-B subunits (FXIII-A2B2). Factor XIII in plasma is a heterotetramer composed of two A subunits arranged as a dimer in association with two B subunits (39). The A subunits are responsible for the transglutaminase activity after the thrombolytic cleavage of an N-terminal activation peptide (40). The B subunit is a glycoprotein and has no known enzymatic activity. It is thought that the B subunit forms a complex with the A subunit dimer and protects it from elimination from the circulation. This view is supported by the observation that genetic deficiency of B subunits leads to a secondary deficiency of A subunits in the plasma (41).

The circulating FXIII-A zymogen is proteolytically activated by thrombin to release a 37 amino acid activation peptide. The presence of Ca2+ in plasma induces conformational changes of the cleaved FXIII-A, which leads to the release of FXIII-B 2 and exposure of the active site cysteines (Cys314) of FXIII-A 2 , thus rendering pFXIII fully active (FXIIIa) (20). Circulating FXIII is bound to fibrinogen, which reduces the threshold level of Ca 2+ required to induce the activation-dependent conformational change of FXIII-A (21). The presence of polymerizing fibrin has been shown to enhance the thrombin cleavage efficiency of pFXIII, which is believed to boost FXIIIa generation during clot formation (22). The binding of fibrin(ogen) and pFXIII probably occurs through FXIII-B 2 , but there is some uncertainty regarding the binding site on fibrin (ogen). Both the c′-splice form of the fibrinogen c-chain and the aC-chain of fibrinogen have been suggested to contain the binding site (21,23–25). This topic has recently been reviewed in detail (26). Following induction of coagulation and cross-linking of fibrin by FXIIIa, the level of non-cross-linked fibrin declines, limiting further FXIII activation possibly to avoid excessive

cross-linking. Furthermore, it has been shown that elastase released by invading and imbedded granulocytes of the hemostatic plug is the primary prote- ase degrading FXIIIa (27). The binding of pFXIII to fibrinogen in the circulation might also ensure efficient delivery of pFXIII into the forming clot where fibrin (ogen) is deposited The circulating FXIII-A zymogen is proteolytically activated by thrombin to release a 37 amino acid activa- tion peptide. The presence of Ca 2+ in plasma induces conformational changes of the cleaved FXIII-A, which leads to the release of FXIII-B 2 and exposure of the active site cysteines (Cys314) of FXIII-A 2 , thus rendering pFXIII fully active (FXIIIa) (20). Circulating FXIII is bound to fibrinogen, which reduces the threshold level of Ca 2+ required to induce the activation-dependent conformational change of FXIII-A (21). The presence of polymerizing fibrin has been shown to enhance the thrombin cleavage efficiency of pFXIII, which is believed to boost FXIIIa generation during clot formation (22). The binding of fibrin(ogen) and pFXIII probably occurs through FXIII-B 2 , but there is some uncertainty regarding the binding site on fibrin (ogen). Both the c′-splice form of the fibrinogen c-chain and the aC-chain of fibrinogen have been suggested to contain the binding site (21,23–25). This topic has recently been reviewed in detail (26). Following induction of coagulation and cross-linking of fibrin by FXIIIa, the level of non-cross-linked fibrin declines, limiting further FXIII activation possibly to avoid excessive cross-linking. Furthermore, it has been shown that elastase released by invading and imbedded granulocytes of the hemostatic plug is the primary prote- ase degrading FXIIIa (27). The binding of pFXIII to fibrinogen in the circulation might also ensure efficient delivery of pFXIII into the forming clot where fibrin (ogen) is deposited The circulating FXIII-A zymogen is proteolytically activated by thrombin to release a 37 amino acid activa- tion peptide. The presence of Ca 2+ in plasma induces conformational changes of the cleaved FXIII-A, which leads to the release of FXIII-B 2 and exposure of the active site cysteines (Cys314) of FXIII-A 2 , thus rendering pFXIII fully active (FXIIIa) (20). Circulating FXIII is bound to fibrinogen, which reduces the threshold level of Ca 2+ required to induce the activation-dependent conformational change of FXIII-A (21). The presence of polymerizing fibrin has been shown to enhance the thrombin cleavage efficiency of pFXIII, which is believed to boost FXIIIa generation during clot formation (22). The binding of fibrin(ogen) and pFXIII probably occurs through FXIII-B 2 , but there is some uncertainty regarding the binding site on fibrin (ogen). Both the c′-splice form of the fibrinogen c-chain and the aC-chain of fibrinogen have been suggested to contain the binding site (21,23–25). This topic has recently been reviewed in detail (26). Following

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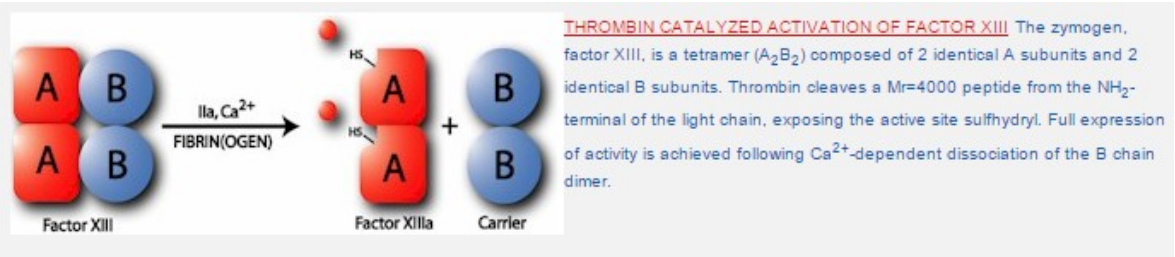


Figure 3. Thrombin catalyzed activation of FXIII

2.1. The role of FXIII in cardiovascular diseases

Beside the role of FXIII in coagulation cascade, it also appears crucial for wound healing and tissue repair, in particolar after MI. Two studies in the late 1970s investigated in detail the changes of FXIII and fibrinogen levels after ischaemic events, ascribing this phenomenon mainly to extra- or intra-vascular coagulation reflecting the degree and duration of the concomitant coagulopathy, but not excluding the possibility that fibrin deposition in the infarcted area could be a pathophysiological reaction to injury and inflammation and a possible repair mechanism (48, 49). Evidences of the essential role of FXIII-A in acute and chronic infarct scar stability come from an experimental animal model with genetically reduced FXII-A levels (50). Authors found that FXIII deficient mice had increased fatal left ventricular wall rupture and dysfunction after MI, whereas FXIII replacement was protective. FXIII thus mediates the formation of a well-cemented scar, essential for structural and functional recovery after MI. In the study of Nahrendorf et al. (51) the research group attempted to translate these animal findings into a potential therapeutic impact of TG modification for human MI. In addition, reduced FXIII activity imaged via single photon emission computed tomography (SPECT)– computed tomography (CT) predicted adverse infarct healing after MI in mice. The importance of

FXIII role in healing cardiac tissue damaged by MI is also stressed by other studies (52, 51, 53), while a therapy based on FXIII supplementation and even intramyocardial injection of FXIII-modifiable biomaterial has been suggested (54, 55). Moreover, myocardial healing, or thrombus formation/fibrinolysis in vivo, can be monitored by using specific FXIII-substrates, allowing non invasive molecular imaging approaches (56, 51, 57). These data support the hypothesis that appropriate levels of FXIII-A, or of its derived by-products, at the injury site is an essential requisite for optimal myocardial healing particularly in the earliest phases. However, no definitive or conclusive results have been produced so far on the extent of FXIII level/consumption as a predictor of the different clinical outcome.

2.2. FXIII polymorphisms

The gene coding for the A subunit of FXIII is localized on chromosome 6p24-25. Previous studies have shown that the A subunit is genetically heterogeneous and a number of polymorphisms have been identified in the protein sequence (58, 49). Several studies have shown that there is a large range of plasma A subunit transglutaminase activity in the normal population, and it is possible that the different levels of activity are related to the inheritance of different allelic variants (59).

2.3. Factor XIII A subunit polymorphisms

A common polymorphism in the FXIII gene— V34L (rs5985)—is one of the most important functional polymorphisms described so far in the hemostatic system. V34L is critically located near the thrombin activation site (R37-G38) of FXIII-A and has been clearly demonstrated that the higher rate of proteolytic truncation of L34 variant resulted in earlier activation of FXIII and, consequently, accelerated the cross-linking of fibrin γ-, and α-chains and the cross-linking of α2-PI to fibrin (60). Moreover, this polymorphism also has a significant effect on fibrin clot structure, probably through the alteration of fibrin cross-linking kinetic. Moreover, turbidometric measurements and electron microscopy confirmed the presence of thinner fibrin fibers and decreased porosity in the presence of L34 (61). In spite of contradictory results, paradoxically the FXIII V34L polymorphism might be a relatively weak protective factor in arterial and venous thrombosis (62). Studies

have reported that the prevalence of the Leu encoding allele is lower in patients with myocardial infarction (63), deep vein thrombosis (64) and cerebral infarction (65) when compared with matched control groups. These clinical studies suggest that this polymorphism may be a risk determinant of thrombosis in both the arterial and venous systems.

P564L constitutes a C-to-T transition at +1694 in exon 12 and alters proline 564 to leucine in barrel 1 with an observed increase in the activity. Recently, this polymorphism has been associated to a decrease in circulating levels of FXIII (66, 67). In fact, case-control studies conducted on a population of women under the age of 45 years have highlighted its involvement in increasing the risk of on fatal hemorrhagic attack, especially when associated with another polymorphism: T204P. Leu564 alters the domain surface that allowing the interaction between subunits A and B. Proline and leucine are amino acids with very different properties, therefore the subunit A structure is sufficiently altered in its efficiency to interacts with subunit B (68). It has been hypothesized that Leu564 variant prevents efficient binding between A and B subunits. This less stable complex would result in lower plasma FXIII levels (67). For these reasons, the Leu564 variant could be important in several clinical events where lower FXIII plasma levels are present, such as Crohn 's disease, ulcerative colitis and in both benign and malignant gynecological tumors (69) .

The T204P variant is characterized by a transversion in exon 5 G→T. The presence of Phe in position 204 is associated,besides reccurent miscarriages, also to a reduction in enzyme activity (70) which can cause a decrease in the haemostatic plug stability, resulting in a high risk condition of intracranial bleeding and ischemic attacks, in particularly in young women constitutionally presenting low FXIII levels (71).

The V650I variant is located in exon 14, originated from a Transition G→A. Although the role of this polymorphism is not yet clear , it could take part in the pathogenesis of diseases thrombotic through its influence of FXIII activity or antigen level subunit A. Recent work has shown that the cardioprotective effect of this polymorphism is attributable to the simultaneous presence allele Leu34 attributing polymorphism Glu651Gln a single role as a marker of the presence of the main functional polymorphism V34L (72, 73).

2.4. Factor XIII B subunit polymorphisms

The polymorphic nature of FXIII-B was demonstrated a long time ago by an isoelectric focusing technique (74). Molecular genetic and biochemical techniques revealed two major polymorphisms in the F13B gene. An A to G transversion within exon 3 (rs6003) leads to a His to Arg amino acid exchange at position 95 in the mature protein (75). The minor allele (Arg95) is relatively rare (7.5%) in the white population, but it represents the major allele (72.5%) among black Africans (76). The p.H95R polymorphism was found to be a risk factor of venous thromboembolism (VTE) (77). The Arg95 allele was associated with an increased risk of mortality after cerebral ischemia of arterial origin (78). In a study by Reiner et al., the homozygous presence of the FXIII-B Arg95 allele lowered the risk of nonfatal MI in postmenopausal women (79).

Recently, a C-to-G change at nucleotide position 29756 in intron K (80). This polymorphism results in an allele-specific splicing product, in which the last 10 amino acids are exchanged by an alternative sequence consisting of 25 amino acids. The variant sequence includes two additional lysine and one glutamic acid residues. These charged amino acids change the isoelectric point of the protein. The polymorphism characteristically occurs in Asians, and the allele frequency in the white population was found to be 14.2% (81).

Mezei et al., (82) found tha the FXIII-B p.H95R polymorphism did not influence the risk of CAS or MI, while the FXIII-B intron K nt29756 G allele was associated with significant protection against CAS and MI in patients with a fibrinogen level in the upper tertile. Interestingly, the protective effect of the intron K nt29756 G allele prevailed only in the presence of the FXIII-A Leu34 allele, and a synergism between the two polymorphisms was revealed. Carriers of the intron K nt29756 G allele had significantly lower plasma FXIII activity and antigen concentration. As FXIII levels in the lower tertile were also associated with significant protection against MI, it is suggested that the protective effect of combined FXIII-B intron K nt29756 G and FXIII-A Leu34 carriership is related to decreased FXIII levels.

3. AIM OF THE STUDY

Myocardial infarction and the consequent loss of fully functional myocardium is today the major aetiology for heart failure. Despite aggressive primary therapy, prognosis remains serious in patients with large infarction and severe left ventricular dysfunction. Thus, it would be highly desirable to influence healing of the cardiac wound to maintain structure and function of the heart.

FXIII is activated by thrombin in the final step of the clotting cascade coagulation and it has a prominent role in cross-linking soluble fibrin to a stable insoluble clot. FXIII-A plays a critical role in generating a stable haemostatic plug, in wound healing, in tissue repair, and in angiogenesis in vivo and in vitro (77, 83, 84, 85). In addition, FXIII-A is present in platelets, monocytes, and macrophages, all components deeply involved in infarct healing (84, 86–88). Experimental evidence in mouse models suggests that FXIII might play a key role in myocardial healing after infarction (50).

To quantify the real contribution of FXIII in this process, and to explore its possible prognostic role, we monitored the FXIII-A subunit levels in 350 acute MI patients during the first six days (d0-d5) plus a control at 30-60 days (d30). A one-year follow-up was performed for all patients. FXIII levels were not only compared to clinical outcomes but also to those of standard cardiac markers. The aim was to evaluate indipendent capability of FXIII measurements to predict the damage due to the infarction and the clinical outcome but also to evaluate if there is ground to further investigate this molecule for a potential therapeutic approach.

Furthermore, common FXIII gene variants (V34L, P564L, T204P, V650I, H95R, nt29756) significantly influence molecular activity. So, in addition, we analyzed all mentioned variants in the framework of the heart muscle healing process and of damage extention, factors directly related to heart failure and, consequently, to survival.

Different FXIII-A dynamics and levels could be utilized as early prognostic indicators during acute MI, toghether with traditional markers of ischemia routinely performed, in patients with suspected or overt heart failure to personalize treatment. Furthermore, they could be an alternative method for biomarker studies aimed to more practical assesment of progression.

In particolar we want to investigate:

- Correlation between different FXIII levels and the onset of different MACEs, with particular interest to those directly caused by an abnormal remodeling in heart failure;

- Different degrees of FXIII consumption in patients in the acute phase (1 st-5ht day), short follow-up (4 weeks) and long follow up (365 days);
- Confirmation of FXIII action and considering its potential prognostic role as "ACTIVE" indicator of post-AMI repairing ability;
- Role played by FXIII genetic variants in determining post-AMI risk.

4. MATERIALS AND METHODS

4.1. Patients

From January 2009 to December 2011, 350 acute MI patients have been recruited (whole group; mean age 68.2± 12.95 years; 72.8 % men) admitted to the Coronary Care Unit (CCU) of the University-Hospital of Ferrara. Acute MI was defined according to the Joint ESC/ ACCF/AHA/WHF Task Force for the Universal Definition of Myocardial Infarction (37), as a rise and/or fall of cardiac biomarkers (cardiac troponin T -cTnT, CKMB measured by mass assay) with at least one of the followings: symptoms of ischaemia, new or presumed new significant ST-segment–T wave (ST–T) changes or new left bundle branch block (LBBB), development of pathological Q waves in the ECG, imaging evidence of new loss of viable myocardium or new regional wall motion abnormality, identification of an intracoronary thrombus by angiography. Patients with ST-elevation myocardial infarction (STEMI) received primary percutaneous coronary intervention (PCI) within 90 minutes of hospital admission, in case of symptoms ≤ 12 h in duration and in case of symptoms lasting 12 to 24 h if pain consisted at the time of admission. Patients with non ST-elevation myocardial infarction (NSTEMI) underwent coronary angiography within 2 to 72 h from hospital admission, according to the ESC recommendations for invasive evaluation and revascularisation of NSTE-ACS. All patients received standard medical therapy according to the ESC guidelines for the treatment of acute MI unless contraindicated, including aspirin, clopidogrel, glycoprotein IIb/IIIa inhibitors (tirofiban or abiciximab), unfractioned or low-molecular-weight heparin, betablockers, statins, renin and/or angiotensin blockers. The baseline demographic, clinical, echocardiographic, and angiographic test results were collected in all patients. The study was approved by the local ethics committee and all patients gave written informed consent to enter the study.

4.2. Blood samples

Blood was collected in Trisodium Citrate Coagulation tubes at admission (d0) and every 24 h for the additional five days (d1-d5) from the acute confirmed MI event. Control samples were drawn at least after 30-days (d30; range: 30–60 days) to have basal FXIII-A levels far from the acute ischaemic event. Additional blood samples (extended time) were not available for the patients under study. To exclude possible further in vitroenzyme

degradation/activation additional comparative samples were drawn in EDTA plus Aprotinin tubes. Plasma was obtained by blood centrifugation (2,500 g x 10 m), and different aliquots were stored at –80 °C.

4.3. FXIII-A level measurements

FXIII-A antigen levels were assessed by means of a Latex Reagent (HemosIL Factor XIII Antigen) which is a suspension of uniform size polystyrene latex particles coated with rabbit polyclonal antibodies, highly specific for the A-subunit of FXIII according to the manufacturer's instructions (Instrumentation Laboratory, Milan, Italy). FXIII-A was tested by Automated Coagulation Analyzer – Instrumentation Laboratory - ACL Futura Plus at all the recruited time considered.

4.4. Genotype Analysis

Blood was collected at admission to avoid loss of cases. Genomic DNA was obtained from peripheral blood, and genotyping for the FXIIIA-V34L and FXIIIB-H95R polymorphisms were performed by PCR-amplification followed by specific restriction enzyme digestion. The forward and reverse amplification primers for FXIIIA were respectively, 5′-CATGCCTTTTCTGTTGTCTTC and 5′-TACCTTGCAGGTTGACGCCCCGGGGCACTA. The underlined base was changed from the native sequence to introduce a restriction DdeI site and the L34-allele results in digestion of the native PCR product (192 bp). The PCR conditions for FXIIIA were as follows: 5 min initial at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 25 s, and 72°C for 60 s. The forward and reverse amplification primers for FXIIIB were respectively, 5′-AAAGACAAGCTTAGTTTCATCATT and 5′-TCTTCAGTTTAGGAAATGAT TCTTAT and the R95-allele alters a restriction NsiI site in the native PCR product (264 bp). The PCR conditions for FXIIIB were as follows: 5 min initial at 94°C, followed by 30 cycles of 94°C for 60 s, 57°C for 60 s, and 72°C for 90 s. All PCR cycles were performed in a Peltier Thermal Cycler apparatus (PTC-200; M. J. Research, Inc., Watertown, MA) and were completed with a 5 min final extension step at 72°C. DNA digestions were performed according to suppliers' instructions and the digested products were analyzed on 8.5% PAGE stained by ethidium bromide.

Confirmation of genotypes was carried out by regenotyping a random selection of samples for each polymorphism investigated.

Genotyping for FXIIIA -P564L, FXIIIA- Y204P, FXIIIA-V650I and FXIIB-nt29756 was performed by Pirosequencing.

Pyrosequencing technology is based on the sequencing by synthesis principle. After successful incorporation of a nucleotide by a polymerase using a single-stranded PCR (or RT-PCR) fragment as template, the released PPi is converted to light by an enzyme cascade: ATP sulfurylase converts PPi to ATP in the presence of APS. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light, which is detected by CCD sensors and seen as a peak in the raw data output. Apyrase continuously degrades unincorporated nucleotides and ATP. The height of each peak (light signal) is proportional to the number of nucleotides incorporated. Sequential addition of nucleotides allows quantitative decoding of the sequence to analyze.

Two primers for target DNA amplification and one primer for sequencing were designed by PyroMark Assay Design 2.0 (Biotage). The 50 base of the forward primer was labeled with a biotin. PCR amplification reactions contained 5 µl 10x Buffer, 4 µl dNTP, 0.25 U Taq DNA Polymerase, 1 ul genomic DNA, and 40 pmol of amplification primers in a reaction volume of 50 µl. PCR conditions were as follows:

V34L: initial denaturation at 95°C/5', followed by 40 cycles of 95°C/30'', 62°C/30'', 72°C/10'', and finally extending 72°C/7'.

P564L: initial denaturation at 95°C/3'; followed by 31 cycles 95°C/30''; 58°C/30''; 72°C/30''; 72°C/5'

Y204P: initial denaturation at 95°C/2'; followed by 30 cycles 95°C/30''; 54°C/30''; 72°C/1'; 72°C/8';

V650I: initial denaturation at 95°C/15'; followed by 45 cycles 94°C/30''; 60°C/30''; 72°C/30''; 72°C/10';

H95R: initial denaturation at 94°C/4'; followed by 34 cycles 95°C/30''; 55°C/15''; 72°C/10''; 72°C/4';

nt29756: initial denaturation at 95°C/2'; followed by 30 cycles 95°C/30''; 53°C/30''; 72°C/60''; 72°C/8';

Following PCR, the fragments were checked by 2% agarose gel electrophoresis. The single-stranded PCR product labeled with a biotin was tested on a PyroMark ID (Biotage) according to the manufacturer's recommendations. (table 1).

SNP	Primer	Sequence	bp
V650I	Forward	5' biotin CCATCCCTGAGATCATCATCAAG 3'	23bp
	Reverse	5' CTCCAGGACCATCCAGGTGTA 3'	21bp
	Sequencing	5' TTTAAAGGATTGGTAAACTC 3'	20bp
P564L	Forward	5' biotin CACAACCGTTACACCATCACA 3'	21bp
	Reverse	5' GCGTCACGTCGAACGTCT 3'	18bp
	Sequencing	5' CCTTCTTGAATTCTGCC 3'	17bp
Y204F	Forward	5' TGGTGTGAAGATGATGCTGTGTA 3'	23bp
	Reverse	5' biotin TCCATAAAAAATTACCCCGATGT 3'	23bp
	Sequencing	5' TGAGAAAGAAAGAGAAGAGT 3'	20bp
nt29756	Forward	5' GTGACAGAGGGCAGTTAAAATATCC 3'	25bp
	Reverse	5' biotin AACGTTGCTTTCACTTCAGACA 3'	22bp
	Sequencing	5' TTTTCTTCATTTTTTTTACA 3'	20bp

TABLE 1. List of FXIII primers used by pyrosequencing

4.5. Follow-up and description of endpoints

The primary endpoint was a composite of major adverse cardiac events (MACE) consisting of cardiovascular death and heart failure (HF) at 30-days and one year. Cardiovascular death includes death resulting from an acute myocardial infarction, sudden cardiac death, death due to HF, death due to stroke, death due to cardiovascular procedures, death due to cardiovascular haemorrhage, and death due to other cardiovascular causes. Cardiovascular origin of a fatal event was established clinically or

at autopsy. An HF event is defined as hospitalisation or an urgent unscheduled outpatient visit for HF, with documented new or worsening symptoms due to HF, objective evidence of new or worsening HF at physical examination and/or laboratory tests, prompting the initiation or intensification of treatment specifically for HF. The cardiovascular events were defined according to the ACC/AHA and ESC guidelines for the management of patients with STEMI, NSTE-ACS and HF and the Standardized Definitions for Cardiovascular and Stroke End Point Events in Clinical Trials for CDISC (Draft Definitions for CDISC August 20, 2014; http://www.cdisc.org/system/files/all/standard/Draft%20Definitions%20for%20CDISC%20August%2020,%202014.pdf).

4.6. Statistics

Continuous data were presented as means ± standard deviation (SD), with the significance of differences judged by t-test. Categorical variables were summarised in terms of number and percentages with the significance of differences judged by Chi-square test. Survival curves were constructed by the Kaplan-Meier method and survival among groups was compared using the LogRank test. Spearman analysis tested correlation coefficients between FXIII-A and cardiac biomarker levels. The recognition of the FXIII-A threshold(s) at any period of time considered (d0-d5) was obtained by means of the Receiver Operating Characteristic (ROC) analysis, utilising the continuous rating scale powered by http://www.rad.jhmi.edu/jeng/javarad/roc/JROCFITi.html and the points for plotting pasted into the Excel program. Probability was considered significant at a level of $p \leq 0.05$.

In order to investigate the association between F XIII and Troponine and CKMB at each time, we performed a multiple regression analysis together with correlation test for each time point. Multivariate regression was evaluated by using FXIII as dependent variable and Troponin and CKBM as independent variables which will be selected for the model through the stepwise procedure. In this method significant variables are entered sequentially; after entering a variable in the model, check and possibly remove variables that became non-significant. It also selects independent variables on the basis of R-squared measure. At each iteration stepwise introduces or discards one variable in the model if

there is an increase or decrease of R-squared respectively. It stops when the inclusion of a variable do not change the value of R-squared.

5. Results

5.1. Patient's characteristics

Table 2 shows the main baseline characteristics of the MI patients under study. The NSTEMI group showed a higher percentage of classical cardiovascular risk factors compared with the STEMI group. Overall, 13.1 % of patients had Killip class > 1 at entry (15.1 % in STEMI vs 8.1 % in NSTEMI; P value = 0.10).

Characteristics	All cases (n=350)	STEMI (n =251)	NSTEMI (n = 99)	P
Age (y, SD, range)	68.2±12.95 (31-80)	67.1±13.50 (31-80)	71.5±11.33 (38-80)	< 0.01
Male (n, %)	255 (72.8)	185 (73.7)	70 (70.7)	NS
PCI (n, %)	313 (89.42)	235 (93.6)	78 (78.8)	<0.0001
Hyertension (n, %)	232 (66.3)	163 (64.9)	69 (69.7)	NS
Dyslipidaemia (n, %)	125(35.7)	80 (31.8)	45 (45.45)	0.02
Obesity (n, %)	40 (10.6)	27 (10.8)	13 (13.2)	NS
Diabetes (n, %)	82 (23.42)	47 (18.7)	35 (35.4)	0.00
Smoking (n, %)	189 (54.0)	140 (55.8)	49 (49.5)	NS
Familiarity (n, %)	115 (32.9)	82 (32.7)	33 (33.4)	NS
Previous MI (n, %)	97 (27.7)	58 (23.2)	39 (39.4)	0.01
Killip class > 1 (n, %)	46 (13.1)	38 (15.1)	8 (8.1)	NS
EF% ≥50 % (n, %)	**142 (40.6)**	**96 (38.24)**	**46 (46.55)**	**NS**
EF (% ± SD)	**44.9 ± 11.27**	**44.56 ± 10.82**	**45.6 ± 12.34**	**NS**

Table 2: Main baseline characteristics in the whole MI group, in the STEMI and NSTEMI subgroups. *PCI= percutaneous coronary intervention. STEMI= patients showing ST-segment elevation MI at enrolment; NSTEMI= patients not showing ST-segment elevation MI at enrolment; EF= Ejection Fraction.*

5.2. Post-AMI FXIII levels and endpoints

The analysis of FXIII levels measured in the plasma of patients at enrollment (t0), after five days to infarction (t1 - t5) , and after a month (t30) provides confirmation of a reduction in FXIII physiological levels of in patients after AMI, in particular between the 4th and 5th day: t0 98.5 ± 31.2 , t4- t5 82.6 ± 3.7, t30 107.2 ± 26.7 , (median values: t0

95.3, t4- t5 82.6 , t30 106.7) (Chart 1). Statistical significance has been assessed comparing t0 vs t4- t5 , P < 0.0001, and t0 vs t30 , P value < 0.0001.

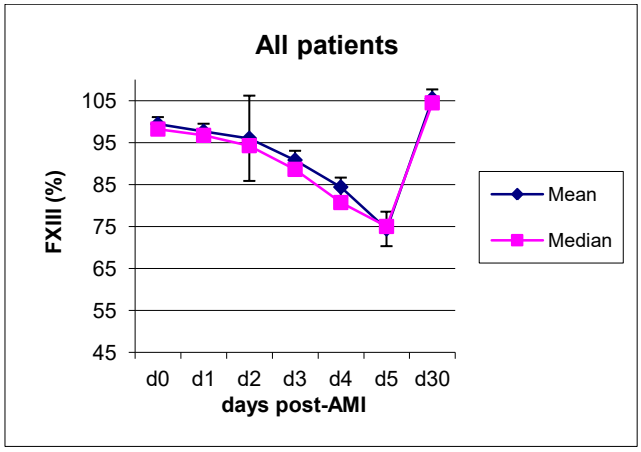


Chart 1. *Kinetics of FXIII levels in the first 5 days and at 4 weeks of post AMI follow up. In blue mean values, in purple, median values.*

Variations in FXIII decrease were evaluated according to the different endpoints considered. Patients developing Heart Failure, as seen in Chart 2, showed a more marked reductions with kinetics: t0 94.4 ± 32.9 ; t4-5 74.7 ± 5.2 ; t30 101.3 ± 29.2 (median values : t0 88.7 t0 , t4 - t5 76.4 , t30 95.9) and Δmean consumption of approximately 20%. Factor XIII levels measured at t4-5 were significantly lower (P value < 0.0001) when compared with the respective baseline values at t0 .

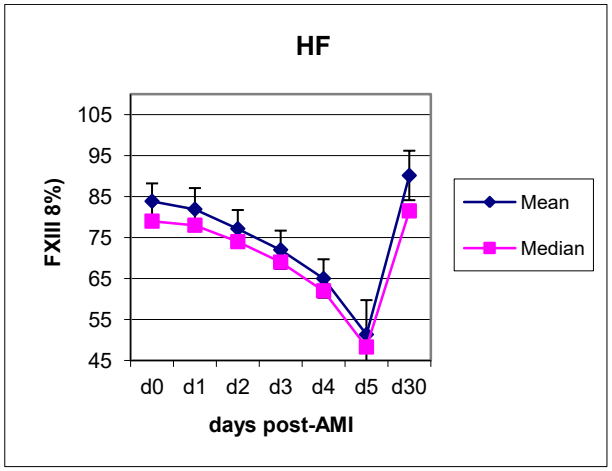


Chart 2. *Kinetics of FXIII levels in the first 5 days and at 4 weeks of post AMI follow up in patients developing Heart Failure. In blue mean values, in purple, median values.*

The unstable angina and RE-AMI did not seem to be affected by lower levels of FXIII. Regarding unstable angina, we did not see consumption peaks, but a gradual lowering maintained to day t30. This is probably due to the fact that angina is a condition of continuous restenosis, consequently FXIII is continuously consumed. Kinetics for angina is: t0 98.9 ± 38.6 ; t4-5 67.7 ± 20:25 ; t30 87.2 ± 7.3 (median value: t0 89.2, t4 - t5 68.5 , t30 87.2). In this case, values at t4-5 lower (P value = 0.04) when referring to values at t0 (chart 3). Considering RE AMI endpoint, the trend appears mainly linear (FXIII % lower average value (t4-5): 94.7 ± 34.8) (chart 4).

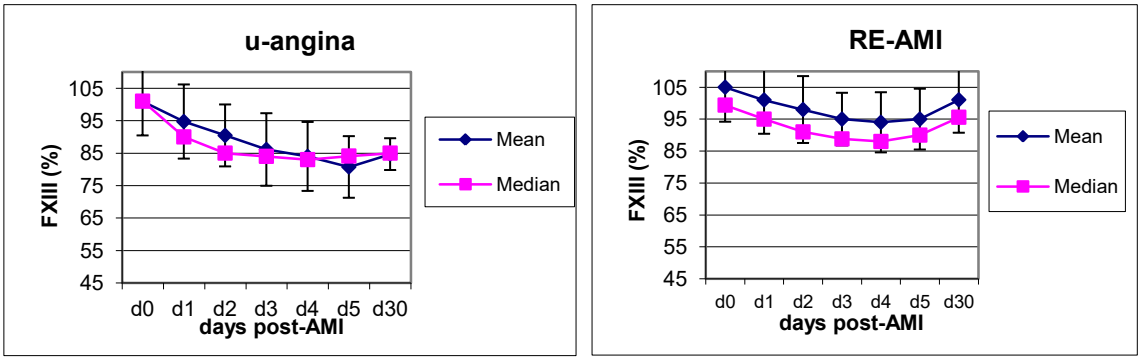


Chart 3 and 4. *Kinetics of FXIII levels in first 5 days and at 4 weeks of post AMI follow -up in patients with unstable angina and reinfarction. In blue mean values, in purple, median values.*

In cases resulting in the death of the patients, a significant lowering of FXIII levels is observed right from enrollment (t0), with percentages mean values of FXIII (t4-5) of 69.5 ± 28.5 (chart 5).

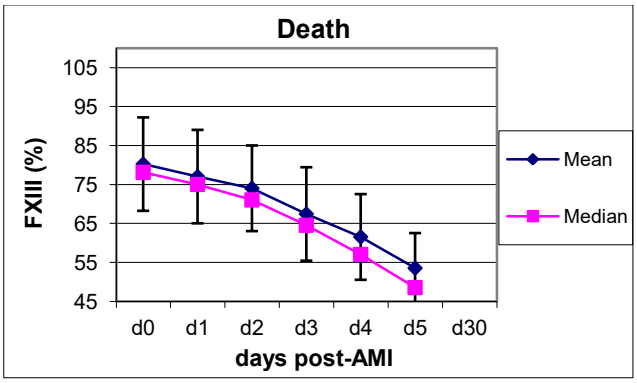


Chart 5. *Kinetics of FXIII levels in the first 5 days post-AMI in death patients. In blue mean values, in purple, median values.*

5.3. Kaplan-Meier survival analysis

Considering the observed decrease after infarction, in particular between 4th and 5th day, survival at one year follow-up was evaluated. Cut-off of FXIII returned from data analysis with ROC curve was 74.5%, [HR365gg 2,62 (1,55-4,43) P value= 0.0004].

Considering as endpoints HF, Stroke, Angina, ReAMI and Death, significant differences in the survival curve have been observed, with an increased survival rate among patients presenting an average percentage of FXIII on the 4th day higher than 74.5% (P value = 0.0004). Patients with lower levels of FXIII after 4-5 days from the ischemic event had the greatest likelihood of developing HF or death by about 1:51 fold (HR = 2:62 ; 95 % CI , 1:55 to 4:43) (Figure 4).

Similar data have been obtained considering as endpoints only HF and death, with higher statistical significante [HR365gg 3,54 (2,07-6,05) P value< 0.0001].

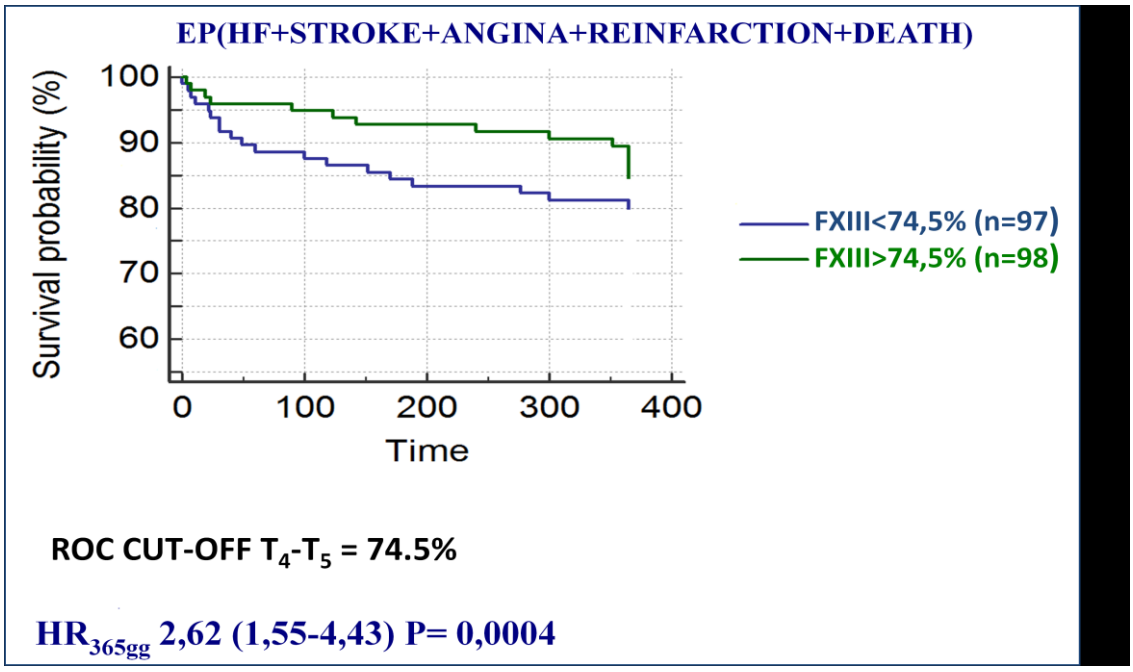


Figure 4. *Survival analysis at 1 year follow-up in the whole group stratified by the cut-off of FXIII = 74.5 % ; P = 0,0004, considering as endpoint HF, Stroke, Angina, Reinfarction, Death.*

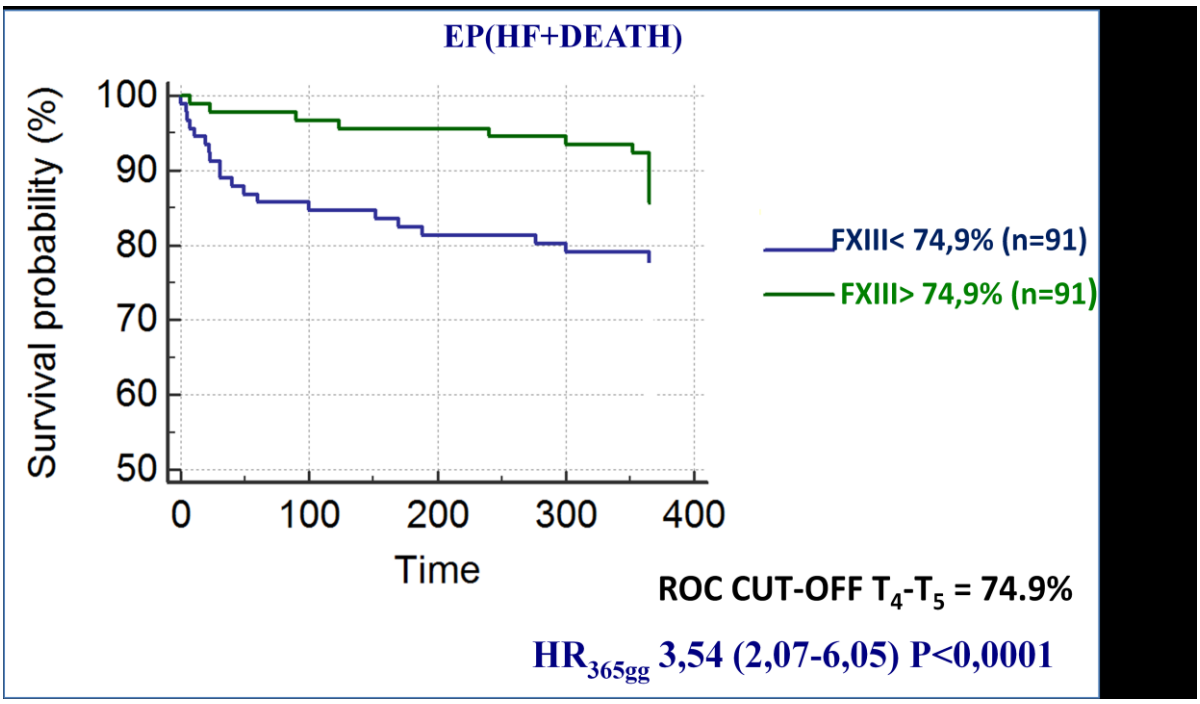


Figure 5. *Survival analysis at 1 year follow-up in the whole group stratified by the cut-off of FXIII = 74.5 % ; P = 0<0001 considering as endpoint HF and Death.*

5.4. Correlation between FXIII levels and serum cardiac biomarkers

In order to deepen investigation of the potential use of FXIII as a novel biomarker of myocardial injury amount, we tested the association between FXIII and Troponin and CKMB, so we performed a multiple regression analysis together with correlation test for each time point. A significant correlation between cardiac biomarkers considered (CKMB and Troponin) and FXIII was observed, in particular, at t3 for FXIII and CKMB (coefficient = 0.154; p value = 0.0107) and for FXIII and Troponin (coefficient = -2.077; p value = 0.0211); at t4 for FXIII and CKMB (coefficient = 0.303; p value = 0.0031) and for FXIII and Troponin (coefficient = -2,198; p value = 0.0448); at t5 for FXIII and Troponin only (coefficient = -3.702; p value = 0.0333). No significant correlation was observed between biomarkers and FXIII levels at t1 and t2 for any correlation and at t5 for FXIII and CKMB (Chart 6).

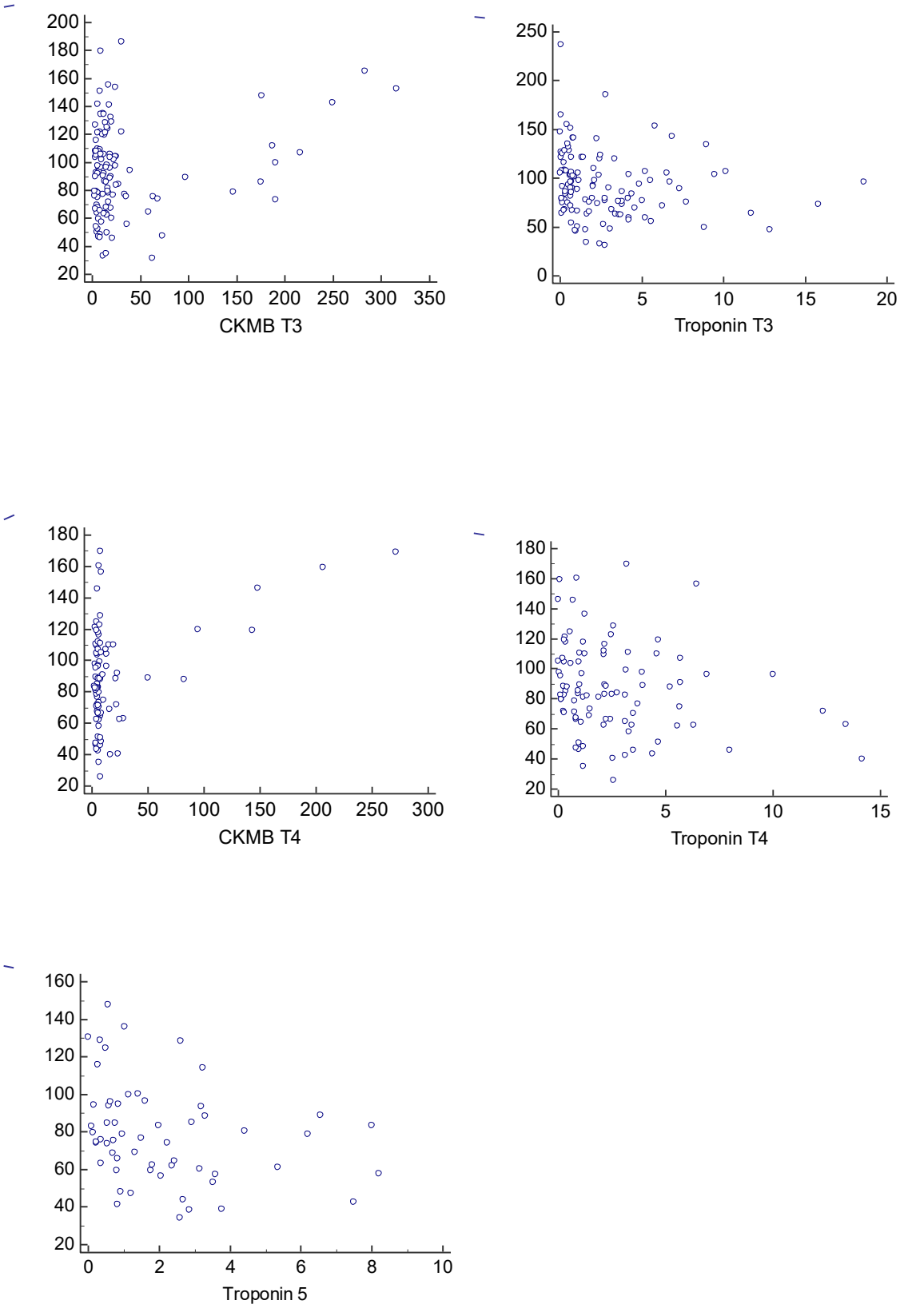


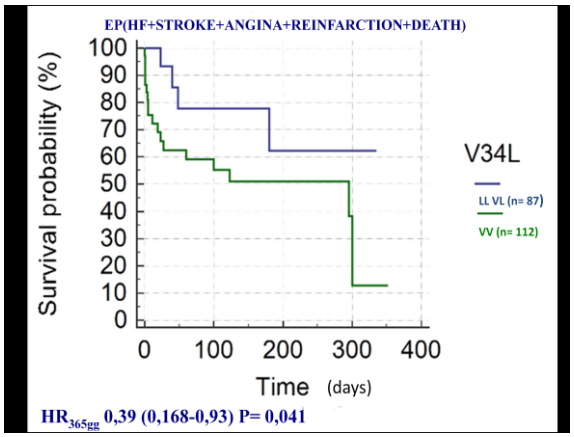
Chart 6. *Plotted correlations between measurements of FXIII levels with CKMB and Troponin in different days after AMI.*

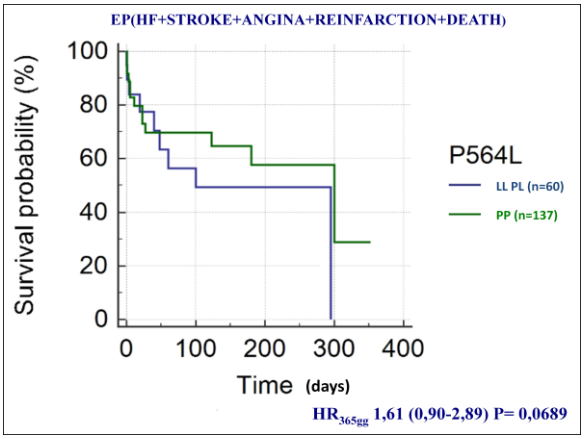
5. 5. Kaplan-Meier survival analysis stratified by genotypes

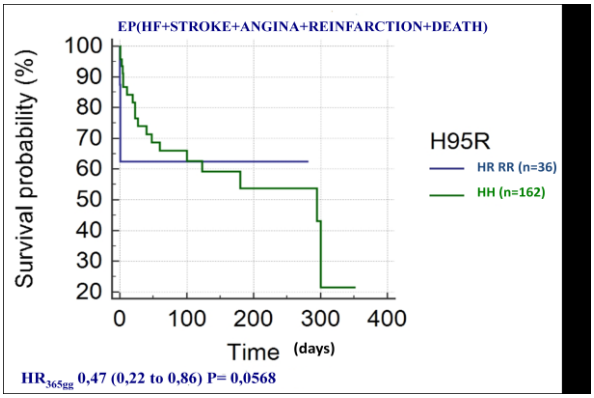
After FXIII levels analysis, we decided to evaluete also the survival rate in the same group of patients considering different genotypes associated to FXIII.

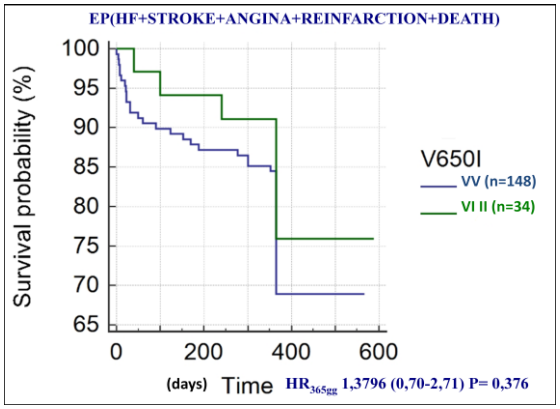
Regarding V34L, LL genotype appears to have a protective role for clinical outcomes when all the adverse events are considered [HR365gg 0,39 (0,168-0,93) P value = 0,041]. This is in agreement with current literature. Actually, the codon 34 is situated at three amino acids from the site for activation by thrombin. When leucine is present, there is an increase of the catalytic activity that increases the speed of clot stabilization and alters the three dimensional structure.

Regarding P564L, H95R, V650I, nt29756 polymorphisms, we did not observed statistical significance, albeit PP variant of P564L shows a protective trend (HR365gg 1,61 (0,90-2,89) P value = 0,0689) (figure 6).









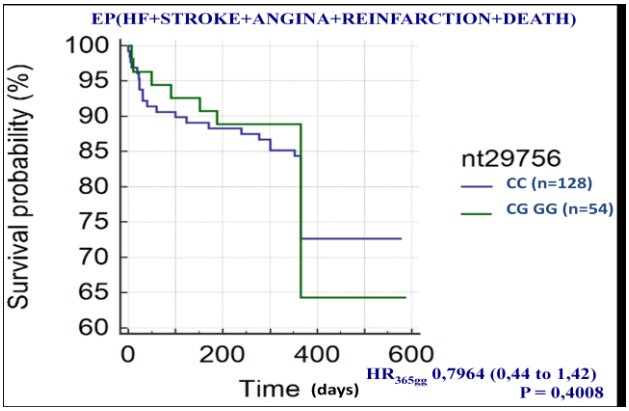


Figure 6. *Kaplan Meier servival analysis for polymorhisms considered.*

6. DISCUSSION

Despite significant progress in the care for patients with MI, the development of post-MI heart failure remains one of the biggest problems in cardiovascular medicine. Prognosis remains poor in these patients, and an early individuation of those at high risk of developing HF could lead to a more adequate and aggressive treatment aimed specifically to them. At the same time, therapies aiming to ameliorate healing process of heart tissue are definitely needed, as very few therapeutic opportunities are available at the present time. Given the involvement of FXIII in the process of wound healing and tissue repair, it has been hypotetized that its activity could represent a new molecular biomarker of heart damage to be added to the ones already in use. Early detection of patients by specific biomarkers may, in fact, help to ensure more aggressive treatment and to improve clinical outcome.

In this framework, we analyzed FXIII in the early days after myocardial infarction in a 350 patients group. The experimental data herein collected showed that an acute (d0-d5) and transient fall in FXIII-A levels virtually occurs, albeit to a different extent, in the whole cohort of MI patients. This is compatible with both coronary thrombus formation, in which activated FXIII-A cross-links fibrin, and the subsequent myocardial healing processes and scar formation.

We focused on the relationship between amount of FXIII depletion and death, unstable angina, reifarction and HF, in the hypotesis that an higher rate of consumption is related to a poorer outcome.

Of particular interest are the data regarding HF and death. Survival analysis has been conducted by looking at heart failure developing or death in patients stratified by FXIII levels. In the case of HF we observed a remarkable lower level of FXIII in patients developing this condition at t4-5, while, in cases resulting in death, a lower level is observed right from t0 and it is maintained throughout time points until t5.

1. These observations strongly suggest that FXIII measurements in post AMI patients could represent a viable mean of assessing their risk of developing HF or of death, thus providing a new tool in order to plan therapeutic interventions.

Natriuretic peptides are commonly used in the evaluation of heart failure, but their role extends beyond diagnosis and includes risk-stratification and management of heart failure patients. We therefore investigated if the FXIII consuming could be dependent on the amount of injury assessed by CKMB or Troponin. For troponin we found significant results at T3, T4 and T5, all of them showing an inverse correlation with FXIII. So, the higher troponin levels, the lower are FXIII levels. This obervation supports the hypotesis of a larger amount of injury in presence of an higher FXIII consumption.

Of uncertain interpretation are the results regarding CKMB. Further studies will be necessary to better evaluate this aspect.

There is substantial evidence that genes play a role in the pathophysiology of heart failure (89). Numerous studies have investigated the association of heart failure with polymorphisms in candidate genes. So far, genetic association studies in heart failure and cardiac remodeling have been highly inconsistent. Since heart failure is a complex trait, there are probably several genetic variants that together result in the expression of its pathological phenotype (90). In this case, we focalized our attention on specific FXIII polymorphisms, located both in subunit A and subunit B, already known to influence molecular. V34L, when genotype LL is present, shows a protective role for reinfarction, as well as for angina, HF, stroke and death. This is not true for other genes considered (T204P, V650I, H95R, nt29756), while P564L, PP variant, shows a similar trend. This observation strongly suggest two possible outcomes: from the prognostic point of view, genotyping of these two genes could lead to a more tailored approach to the post AMI patients. From a therapeutic point of view, since FXIII supplementation is currently being considered as a potential treatment in post AMI (50), utilization of a FXIII variant with an higher protective role could lead to more efficient implementation of this therapeutic approach aimed to improve healing process in heart tissue.

7. CONCLUSIONS

Considering relationship between FXIII levels and outcomes in post AMI patients, it could represent a strong candidate as prognostic factor, with the possibility of using it togheter with already established biomarkers in order to precisely define high risk patients. This will lead to a more tailored approach for therapeutic interventions.

Furthermore, the role of circulating FXIII in maintaining myocardium structural integrity opens up good expectations. Mice KO for FXIII gene died due to heart rupture after experimental AMI. FXIII, by transforming fibrin biopolymers in an elastic bio-scaffold, could promote recruitment, attachment and differentiation of resident and circulating SCs leading to neoangiogenesis, cardiomyocytes survival and decreased fibrosis. FXIII treatment in animal model of AMI could test this hypothesis. Finally, the observation of specific FXIII polymorphisms with a protective role could pave the way to more effective supplementation therapies.

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