Universitat Autònoma de Barcelona

ANALYSIS OF THE LEVELS OF MONOCYTE SUBSETS IN PATIENTS WITH HEART FAILURE

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Programa de Medicina

Departamento de Medicina. Facultad de Medicina

ANALYSIS OF THE LEVELS OF MONOCYTE SUBSETS IN PATIENTS WITH HEART FAILURE

DOCTORAL THESIS

Presented by

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El Dr. Antoni Bayés Genís, Director del Instituto del Corazón Hospital Universitario Germans Trias i Pujol de Badalona y Profesor Titular del Departamento de Medicina de la Universidad Autónoma de Barcelona; y el Dr. Josep Lupón Roses, Jefe de Sección del Servicio de Cardiología y Coordinador de la Unidad de Insuficiencia Cardiaca del Hospital Universitario Germans Trias i Pujol de Badalona, y Profesor Asociado del Departamento de Medicina de la Universidad Autónoma de Barcelona,

CERTIFICAN: Que Elena Georgieva Elchinova, Licenciada en Medicina, ha realizado bajo su Dirección la Tesis titulada "ANALYSIS OF THE LEVELS OF MONOCYTE SUBSETS IN PATIENTS WITH HEART FAILURE" / "ANALISIS DE LOS NIVELES DE SUBPOBLACIONES DE MONOCITOS EN PACIENTES CON INSUFICIENCIA CARDIACA", para optar al Grado de Doctora y que la mencionada tesis cumple todos los requisitos necesarios para ser defendida en el Tribunal correspondiente.

Badalona, 20 de mayo de 2019

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DEDICATION

Dedico esta tesis doctoral a todas las personas con insuficiencia cardíaca que depositan en nosotros su confianza y fe para buscar un alivio para su enfermedad y esperan ser atendidos de una manera profesional y humana.

This doctoral thesis is dedicated to all patients with heart failure that believe in us to find relieve for their disease and expect to be attended in a professional and human way.

AGRADECIMIENTOS

Siempre es difícil iniciar un Proyecto desde un principio. Desde el momento cero aparecen muchos inconvenientes y preguntas abiertas que nadie se ha imaginado que podrían suponer un problema. Sin embargo, un equipo unido y abierto para la discusión y la ayuda, como ha sido el mío, siempre encontrará el camino para que el proyecto llegue a su fin. Por eso, presentando yo la tesis, no quiera decir que la he hecho sola. Aquí está reflejado el trabajo durante muchos años de personas muy válidas e inteligentes que han puesto su esfuerzo en desarrollar y acabar este proyecto.

Muchas son las personas a las cuales tendría que agradecer haberme ayudado. Un papel primordial han tenido las enfermeras de la Unidad de Insuficiencia cardiaca del Hospital Germans Trias i Pujol que, a pesar del enorme trabajo que tienen que hacer a diario atendiendo de una manera de lo más profesional y humana nuestros pacientes de insuficiencia cardiaca, han podido encontrar el tiempo de dedicarse también a la investigación que suponía el proyecto.

Me gustaría destacar dos personas, mis directores de la tesis, Dr. Josep Lupón y Dr. Antoni Bayés Genís, dos cardiólogos excelentes y líderes a nivel nacional e internacional en el campo de la Insuficiencia cardiaca, que ocupan un lugar especial en este proyecto, siendo las personas que siempre han tenido una enorme paciencia conmigo, me han apoyado en todos y cada uno de estos momentos durante seis años y siempre me han ayudado en todo lo que ha estado en sus manos para que yo pueda llevar a cabo este proyecto.

A Iris Teubel, la técnica del proyecto que con el tiempo se ha hecho una amiga, a Santi y Marco, los investigadores del centro de Investigación clínica del Germans Trias i Pujol le doy mi enorme gratitud por estar siempre allí, por su dedicación al proyecto y enorme trabajo.

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ABBREVIATIONS LIST:

NT-proBNP: N-terminal pro B-Typ natriuretic peptide

BNP: brain natriuretic peptide

ANP: atrial natriuretic peptide

CNP: C natriuretic endothelial peptide

NYHA functional classification: New York Heart Association functional classification

ST2: suppression of tumourigenicity 2 (Interleukin-1 receptor-like 1)

IL: Interleukin

KDR: kinase insert domain receptor

SEB: Staphylococcal enterotoxin B

MCH class II: Major histocompatibility complex class II

LVEF: the left ventricle ejection fraction

flow-FISH: fluorescent in-situ hybridization

BB515: Brilliant Blue

PI staining: Propidium iodid staining

LPS: Lipopolysaccharide

ACE: Angiotensin converting enzyme

TNF alpha: Tumour necrosis factor alpha

RNA: ribonucleic acid

DNA: deoxyribonucleic acid

BRIEF PRESENTATION OF THE THESIS

The present doctoral thesis is presented in form of original article compendium, being approved in first place from the Academy Commission of the Doctoral Programme of the Autonomous University of Barcelona, where the doctoral aspirant is main author or co-author of all the articles.

The fundamental part of the work is centred in the study of the monocyte levels, distribution and clinical significance in outpatients with heart failure of a multidisciplinary Heart failure Unit of the University Hospital Germans Trias i Pujol in Badalona. It was investigated if the levels and distribution of the different monocyte subsets could be used as a prognostic predictor factors in Heart failure. Additionally, on one hand, the doctoral thesis describe an innovating method of the simultaneous analysis con the relative telomere length and distribution of the monocytes and on the other hand, if the relative telomere length could be related with adverse events (mortality of hospitalizations) due to heart failure.

This investigation has been financiered by grants from the Ministerio de Economía, Industria y Competitividad, the Fundation La MARATO de TV3, CIBER Cardiovascular, Red de Terapia Celular-TerCel, AdvanceCat with the support of ACCIO (Catalonia Trade & Investment; de Catalunya) under the Catalonian European Regional Development Fund operational program (2014-2020), the Bank Fundation La Caixa, and Fondo de Investigación Sanitaria, Instituto de Salud Carlos III.

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1. SUMMARY

1.1. SUMMARY

Heart failure is a disorder characterized by different clinical signs and symptoms due to a structural or functional anomaly of the heart. It is the most predominant heart disease in developed countries, both from epidemiological point of view and clinical implications. Indeed, it is a growing medical problem related to major hospitalization needs and high mortality, with significant economic and population burden worldwide. Established prognostic factors, such as age, sex, aetiology, comorbidities, New York Heart Association functional class, left ventricle ejection fraction, and routine laboratory markers might fail to completely and individually predict disease progression and mortality. A good risk stratification strategy is crucial as risk might be refined using several biological biomarkers of different pathophysiological processes that the former mortality risk factors do not necessarily directly reflect. That is why efficient and reliable new prognostic predictor markers are of upmost importance and relevance for the future management of the disease.

Monocytes are a heterogeneous population of effector cells with key roles in the maintenance and restoration of tissue integrity. Three distinct human monocyte subsets can be identified by flow cytometry: classical (CD14++/CD16–), intermediate (CD14++/CD16+) and non-classical (CD14–/CD16+). Little is known about the importance, relationship between the levels of the circulating monocytes and their distribution in heart failure, even less if these parameters could be used as a predictor markers for the progression of the disease.

The main objective of the current project was to assess the relationship between the levels and distribution of the different circulating monocyte subsets and the length of its telomeres in outpatients with heart failure with adverse events, namely mortality and heart failure hospitalizations.

Three cohorts of respectively 28, 400 and 101 ambulatory patients, consecutively treated at a multidisciplinary heart failure Clinic from December 2013 to May 2015 were included in the studies described in this doctoral thesis, independently of the data of their entry into the heart failure Clinic program. All study procedures were performed in accordance with all ethical standards and all participants provided written informed consent. Peripheral blood samples of all patients were extracted for subsequent analysis by flow cytometry. The samples were incubated directly by means of monoclonal antibodies with fluorocromes against monocyte specific surface antigens, type CD86 (or HLA -DR), CD14 and CD 16 and in parallel (in 100 samples) genetic markers (telomeres) were subsequently analyzed by flow cytometer (BD LSRFortessa) in the Department of Citolatry of the IGTP. The percentage distribution of each monocyte subset was analyzed and their absolute cell count (U/mL) was also determined quantitatively. We were able to establish an innovating, accurate and much less expensive method than established ones for simultaneously measuring the different monocyte subsets and the its relative telomere length.

In our study, the intermediate subset was independently associated with all-cause death and the composite end-point of all-cause death or heart failure hospitalization, in multivariable analyses. The quantitative determination of the absolute cell count of each monocyte subset expressed by U/mL was superior from the prognostic point of view than the percentage of these monocyte subsets in outpatients with Heart failure. We observed about 22% reduction in telomere length over 1 year in the monocytes of our patients, being the baseline telomere length and change in telomere length not significantly associated with outcomes. Therefore, the change in telomere length is not likely to be a useful biomarker of heart failure progression.

The monocytes and monocyte subsets could be used not only as a predictor factor but also might be taken into consideration as part of an immuno-modulation therapy in the future for the heart failure patients.

1.2. RESUMEN

La insuficiencia cardíaca es un síndrome, caracterizado por diferentes signos y síntomas clínicos debidos a una anomalía estructural o funcional del corazón. Es una de las cardiopatías más predominantes en los países desarrollados, tanto desde el punto de vista epidemiológico como de sus manifestaciones clínicas. La insuficiencia cardíaca es un problema médico creciente relacionado con una alta tasa de hospitalización e importante mortalidad y un pronóstico desfavorable con coste socioeconómico muy elevado en todo el mundo.

Los monocitos son una población heterogénea de células efectoras con funciones clave en el mantenimiento y la restauración de la integridad del tejido y del sistema inmunológico. Mediante citometria de flujo se pueden separar tres subpoblaciones de monocitos humanos distintos: clásicos (CD14 ++ / CD16–), intermedios (CD14 ++ / CD16 +) y no clásicos (CD14– / CD16 +).

Poco se sabe acerca de la importancia, la relación entre los niveles de los monocitos circulantes en sangre periférica y su distribución en la insuficiencia cardíaca, incluso menos se conoce si estos parámetros podrían usarse como marcadores predictores de la progresión de la enfermedad.

El objetivo principal del proyecto actual fue evaluar la relación entre los niveles y la distribución de las diferentes subpoblaciones monocitarias y la longitud de sus telómeros en pacientes con insuficiencia cardíaca y los eventos adversos, comomortalidad y hospitalización por insuficiencia cardíaca. La tesis doctoral actual describe tres estudios, respectivamente de 28, 400 y 101 pacientes ambulatorios, tratados consecutivamente en una unidad multidisciplinaria de insuficiencia cardíaca desde diciembre de 2013 hasta mayo de 2015. Todos los procedimientos del estudio se realizaron de acuerdo con todos los estándares éticos y todos los participantes proporcionaron un consentimiento informado por escrito. Se extrajeron muestras

de sangre periférica de todos los pacientes para su posterior análisis mediante citometría de flujo. Las muestras se incubaron directamente con anticuerpos monoclonales con fluorocromos contra antígenos de superficie específicos de monocitos, tipo CD86 (o HLA -DR), CD14 y CD 16, y en paralelo (en 101 muestras) se analizaron marcadores genéticos (telómeros) mediante un citómetro de flujo. (BD LSRFortessa) en el Departamento de Citolatría de la IGTP. Se analizó la distribución porcentual de cada subconjunto de monocitos y también se determinó cuantitativamente su recuento de células absoluto (U/mL).

Durante nuestro proyecto pudimos establecer un nuevo método de análisis conjunta de subpoblaciones de monocitos y con determinación de la longitud relativa de los telómeros, que resulto rápido, preciso y mucho más barato que otros métodos utilizados previamente.

En nuestro estudio, la subpoblación intermedia se asoció de forma independiente en el análisis multivariable con mortalidad por todas las causas y con la variable compuesta (mortalidad por todas las causas o ingreso por insuficiencia cardiaca). La determinación cuantitativa del recuento de células absoluto de cada subpoblación de monocitos expresado en U/mL fue superior desde el punto de vista pronóstico al porcentaje de estas subpoblaciones. Se observó una reducción de aproximadamente el 22% en la longitud de los telómeros durante un año en los monocitos de nuestros pacientes, aunque la longitud relativa y el cambio en la longitud de los telómeros no se asociaron significativamente con los resultados. Por lo tanto, no es probable que el cambio en la longitud de los telómeros sea un biomarcador útil de la progresión de la insuficiencia cardíaca.

Los monocitos y las subpoblaciones de monocitos podrían usarse en el futuro no solo como un factor predictor, sino que también podrían tomarse en consideración como parte de una terapia de inmunomodulación para los pacientes con insuficiencia cardíaca.

2. INTRODUCTION

2.1. General concepts and relevance of heart failure. Importance of predictor factors and new methods for patients' stratification

Heart failure is a human disorder characterized by different signs and clinical symptoms due to a structural or functional anomaly of the heart. It is one of the most predominant heart diseases in developed countries, with estimated prevalence from 1-2% of the adult population, rising up to >10% among people >70 years of age, being the lifetime risk for heart failure at age of 55 years of 33% for men and 28% for women[1]. It is a growing medical problem related to major hospitalizations, mortality and poor prognosis with significant economic and population burden worldwide. The diagnosis of heart failure is done once clinical symptoms become apparent, not in stages when asymptomatic structural or functional abnormalities of the heart are detected, although these can be a precursor of the heart failure process. Even until now, the diagnosis of heart failure is based on the clinical manifestation, history and examination and there is not one single test that can diagnose heart failure for itself[2,3]. Symptoms of heart failure often are unspecific or could be mistaken with other problems and symptoms, such as fluid retention, could rapidly resolve with adequate treatment. Moreover, specific signs of heart failure could be difficult to detect and even more difficult to reproduce[1]. That is why efficient and reliable diagnostic and moreover prognostic predictor factors are of upmost importance and relevance for the future development and consequent early treatment of the disease. The vital importance and social economic impact of early risk stratification has been supported in other studies in heart failure[4], where establishing an early and accurate diagnosis of heart failure was related with decrease length of hospitalization and costs.

A good biomarker should be accurate and able to be reproduced in different measurements by different operators, it should provide new information, which cannot be obtain with a careful clinical assessment and should help in the decision making for the therapeutic strategy of the

patients[5]. The World Health Organisation proposed a definition for a biomarker as any substance, structure, or process that can be measured in the body or its products and influences or predicts the incidence of outcome or disease[6]. Although many of the common used predictor factors do not fulfil completely these criteria, they are still largely used in the clinical practice. In the clinical trial of Bouvy[7] was developed a prognostic model for the prediction of the mortality risk in patients with moderate and severe heart failure. They studied some parameters such as clinical history, physical examination (blood pressure), drug use, quality of life were in order to stratify the patients according to their short-term risk to death. The strongest predictor factors in this study were the NYHA classification, the Minnesota heart failure score, renal dysfunction and the use or non-use of beta-blocker agents. However, these predictors factors, although easy to obtain and very accurate for the prediction of mortality, did not established the individual risk.

In another article, Braunwald[8] categorized the Biomarkers in heart failure in six main categories (Biomarkers for inflammation (CRP, TNF alfa, IL 1,6-18), Oxidative stress (Oxidized low-density lipoproteins), Etracellular matrix remodelling (a marker for progression of heart failure), Neurohormones (Norepinephrine, Renin, Angiotensin II, Aldosterone, Endotelin), Myocyte injury (Cardiac specific troponin I y T, Myosin light chain kinase I, Creatine kinase MB fraction), Myocyte stress (Brain natriuretic peptide: BNP, N-terminal pro-brain natriuretic peptide, ST2). The study of biomarkers of inflammation in heart failure has been of significant importance in the last decade since the process of inflammation has been related with the progression and severity of the disease.[9] Some of the studied biomarkers are described below:

- CRP

In this context, CRP has proof to be a useful parameter and its high levels have been found in inflammatory conditions (sepsis) as well as in heart failure patients, where it bestowed worse prognosis[8]. At vascular level, CRP reduce the nitric oxide and increase endoteline-1 production

and stimulates the expression of endothelial adhesion molecules. Nevertheless, CRP can be elevated in very heterogenic conditions such as acute and chronic infection, cigarette smoking, acute coronary syndromes, active inflammatory states, etc.

- Cytokines

Another pro-inflammatory agents are TNF alpha and Interleukins 1, 6 and 18, which consequently can elevate after ischaemic cardiac injury with inflammatory stress with the acceleration of progression of heart failure. These interleukins may cause myocyte apoptosis, necrosis, and secondary ventricular remodelling due to matrix metalloproteinase.

- Oxidative stress

The oxidative stress and oxidative particles such as superoxide anion, hydrogen peroxide and hydroxide radical can impair the normal endothelial function and influence the progression of heart failure[8]. It can also lead to cellular damage, apoptosis and necrosis of the myocytes and cause as well cardiac arrhythmias.

- Renin-Angiotensin-Aldosterone

Other type of biomarkers associated with worse prognosis in heart failure are the neurohormones from the Renin-angiotensin aldosterone system, as well endothelin 1 and norepinephrine. The activation of this system together with the sympathetic nervous system in heart failure occurs as a response mechanism to arterial underfilling and other pathophysiological processes (eg. dehydration or blood loss)[10]. This mechanism initially has a positive effect in the homeostasis of the organism but with time can provoke maladaptive cardiac remodelling and progression of heart failure. Although, in clinical everyday praxis their plasma levels are unstable and difficult to measured, their inhibitors as well as the beta-blockers (with blockade of the sympathetic nervous system), play a main role in the treatment of heart failure.

- Natriuretic peptides: ANP, BNP and NT-proBNP

There are different types of natriuretic peptides: ANP (atrial natriuretic peptide), brain natriuretic peptide (BNP) and C natriuretic endothelial peptide (CNP). They are hormones, with diuretic characteristics and who participate in the vasomotoric regulation (stimulating the vasodilatation) and are secreted from cardiomyocytes in response to an atrial or ventricular wall stretch due to volume expansion or pressure overload. The ANP is primary secreted from the atria in form of pro-hormone (pro-ANP), which after been fragmented from a special enzyme, is converted in the active molecule ANP. The BNP is secreted principally from the ventricles in form of pre-hormone and which is disintegrated into NT-proBNP and active peptide BNP. The terminal fragment of the molecule (NT-proBNP) is much more stable and easier measured in comparison to BNP in plasma which explained the reason for its common used in laboratory. BNP promotes vasodilatation, natriuresis, diuresis and inhibits the renin-angiotensin-aldosterone system.

These peptides elevate in patients with heart failure and especially BNP and NT-proBNP are directly proportional with the volume expansion, myocardial wall stress and intracardial pressure and are related to cardiac disorder. The natriuretic peptides are also elevated in states of renal insufficiency, chronical lever or hormonal diseases (Cirrhosis, Cushing syndrome) and acute events such as myocardial infarction, pulmonal embolism/disease or cerebral haemorrhage. They are influenced by the age, sex, special medication, hormones and assay used. The natriuretic hormones and their importance and utility have been investigated in other studies[11,12,13], where sustained plasma levels of BNP were established as reliable and independent risk factor and predictor of death or deterioration of cardiac functional status in heart failure. Monitoring levels of BNP/NT-proBNP is a useful tool to assess the response of medical treatment in acute heart failure decompensation and in outpatients. The natriuretic peptides are also useful for the diagnosis of heart failure, for if their value is below the cutting

point, the diagnosis of heart failure is unlikely and these patients would not require an echocardiography[1].

- ST2 (Interleukin-1 receptor-like 1)

ST2 is other laboratory marker used in the stratification of heart failure. It is part of the interleukin-1 receptor family and is secreted by monocytes subjected to mechanical strain. It appears to decrease the inflammatory response of the organism by suppressing the production of inflammatory cytokines (IL 6 and 12) and its levels are elevated in patients with heart failure[6]. An advantage of this marker is that it is not influenced by age, renal function or body-mas index.[10,14] Soluble ST2 is associated with worse prognosis in heart failure and increase risk of adverse outcomes[10]. Furthermore, the addition of ST2 together with NTproBNP substantially improves the risk stratification for death beyond that of a model that is based only on established mortality risk factors[15]. Indeed, it has been suggested that ST2 may be regarded as the new gold standard biomarker for prognosis and monitoring in heart failure.[16]

- Troponin

The markers for cardiac necrosis, as positive Troponin have shown also to be related to worse prognosis in heart failure as they had more adverse events, increased in hospital mortality, more cardiac procedures and longer hospitalization and consequently they need to be undergo more intensive monitoring and close follow up[17]. In the same study, patients with negative troponin values had two thirds lower mortality rate than the ones with positive troponin. High-sensitivity Troponin T provided significant prognostic information in a Spanish real-life cohort of patients with chronic heart failure, while simultaneous addition of high-sensitivity Troponin and NT-proBNP into a model that included established risk factors also improved mortality risk stratification.[18]

- MicroRNAs

Short, non-coding sequences of RNA that regulate gene expression at the posttranscriptional level and are stable in blood. They have been investigated as possible biomarkers in cardiovascular diseases. Changes in specific microRNAs (29a, 1, 21 and 133a) were associated with myocardial fibrosis[10]. However, these markers are still not used in the clinical practice, as standardized approaches and quality assessment for measuring circulating miRNAs are not uniformly established, and most studies have been small, so the obtained results are inconsistent. In two independent cohorts totalling 2203 subjects higher levels of miR-1254 and miR-1306-5p were significantly associated with risk of the combined endpoint of all-cause mortality and heart failure hospitalization. However, these two circulating miRNAs failed to improve prognostication over established predictors.[19]

- Markers of renal dysfunction (creatinine, estimated glomerular filtration rate and cystatin C)

Markers of renal function have been extensively related to prognosis in heart failure patients. Estimated creatinine clearance and estimated glomerular filtration rate, independently of the equation used for their estimation have shown a very important prognostic value.[20,21] The prognostic role of Cystatin C is more controversial[22].

Other biomarkers described in heart failure, such as Galectin-3, GDF-15, ceruloplasmin, are currently investigated as predictor markers for the development and prognosis of heart failure.[10,23,24]

In the last years numerous prognostic markers have proven to be of significant utility for the diagnosis of heart failure, although their clinical applicability remains limited and diagnosis and therapy monitoring and guidance of heart failure is still challenging.

Usually, prognostic markers are considered separately, but a multimarker strategy could eventually supposed better risk stratification and therefore optimization of the medical treatment and decrease the socio-economic costs.

Diagnosis, progression and prognosis as well as risk stratification of heart failure patients and optimal prediction of adverse cardiovascular events and mortality has been of interest of many investigators groups, who have also suggested practical clinical models and scores[10,26-28] combining different laboratory, clinical, anthropological and imaging parameters. As an example, the Seattle Heart failure score[26] established that the NYHA functional class, ischemic aetiology, diuretic dose, ejection fraction, sodium, haemoglobin, lymphocytes, uric acid and cholesterol have independent predictive power in heart failure. An important limitation of these models is that they help predict death in heart failure patients but are less accurate to predict further hospitalizations due to heart failure.[25,29] Biomarkers have been added into such risk models in recent years. The Barcelona Bio-HF Calculator is a contemporary web-based heart failure risk score derived from a well-characterized and managed, current real-world cohort that combines clinical data (age, sex, NYHA functional class, left ventricular ejection fraction, serum sodium, estimated glomerular filtration rate, hemoglobin), treatments and 3 heart failure biomarkers (i.e., NT-proBNP, surrogate for myocardial strain, high-sensitivity troponin T – surrogate for myocyte damage, and ST2 reflective of inflammation and extracellular matrix remodelling), and it is designed to run with the availability of 0, 1, 2, or 3 of them. It was first developed for calculating the risk of all-cause death up to 3 years[25] and was validated in a Boston cohort from the PROTECT study[30] and included in the references 2016 Guidelines of the European Society of Cardiology[1]. Indeed, an updated version has been more recently developed which allows estimating the risk up to 5 years of all-cause death, heart failure hospitalization (using competitive risks assessment) and the composite of both events[31].

Biomarkers for stratification and progression of disease as well as to measure the response of therapy are of crucial importance in the assessment of acute heart failure, where they could confirm dynamic changes in hemodynamic and clinical status.

A significant limitation of a new and accurate biomarker is the financial side. Due to elevated costs, less developed countries cannot afford optimal medical assessment, including biomarkers.

The described above is only an example, that shows the necessity for the development of new and more precise predictor markers to better understand the human heart and to treat our patients more accurately and safely.

2.2. Definition, characteristics and physiology of monocytes

The monocytes are white blood cells, which are part of the immune system and participate in the homeostasis of the organism and tissue repair. They are generated from precursor cells in the bone marrow and circulate in the blood stream from where, due to specific stimuli, they migrate to the tissues and transform themselves into specific cells called macrophages. They possess only one nucleus, from where comes their name: mononuclear phagocytes or monocytes/macrophages. Their identification occurs based on the cell morphology, cytochemistry as well as flow cytometry. In dependence of the expression of these specific cell-surface markers, monocytes are divided into three different subsets: the classical: with high expression in CD14 and low in CD 16: CD14++/CD16-, the non-classical: low in CD 14 and high in CD16: CD14+/CD 16++ and an intermediate subpopulation: CD 14++/CD16^{+}[32].

The three monocyte subsets differ not only in the expression of surface receptors markers but also in the type of cytokines that they produce.[33,34] This characteristic helps as well to

understand the functional differences between the subsets. For instance, TNF alpha is produced mainly from the CD 16+ subsets (intermediate and non-classical subsets), which has been associated with pro-inflammatory conditions, although some discrepancies about this topic have been described in previous studies[34].

The classical monocyte subset was well known in comparison to the non-classical (CD16+), which was discovered about 20 years ago and accomplished for about 10% of the monocytes. It took even more time to distinguish the third monocyte subset, the intermediate because of the difficulty to distinguish the boundaries between this subset and the other two being a continuum, and to categorize it into a separate subset with complete different functions and secretion of interleukins[32]. The classical monocyte subset acts as scavenger toward apoptotic cells and participate in the resolution of inflammation; it expands in variety of diseases as inflammatory disorders (rheumatoid arthritis, inflammatory bowel disease, severe asthma and kidney insufficiency.[35,36,37]. This monocyte subset segregates different cytokines (interleukin IL-1) associated with an inflammation state and, together with other peripheral blood cells and agents, could produce cardiac damage. The key role of the monocyte subsets in the inflammation and innate and adapted immunity has been researched also in the pathomechanism of the cardiovascular diseases [38-43]. The classical subset can acquire T-cell inducer proliferate activities if properly activated[44].

The same study also describes an important pro-angiogenic capacity of the intermediate subset, based on the expression of proangiogenic factors as endoglin tyrosine kinase and kinase insert domain receptor (KDR). The intermediate subset has been also associated with pro-inflammatory characteristics, and has the ability to respond direct to microorganism.[45,46] It expands with cytokine treatment and inflammation. During infection it is the first subset of the monocytes which would increase in number, followed from the non-classical monocytes. In the last years, the intermediate subset has gained more attention due to its unique characteristics

and clinical importance in chronic inflammation diseases.[46,47] Another interesting point is the capacity of the intermediate subset in expressing high levels of major histocompatibility complex (MCH) class II and the presentation of genes, which makes this monocyte subset one of the best inducer of Staphylococcal enterotoxin B (SEB) mediated T cell proliferation and interaction[44].

In generalized infections with systemic affectation like sepsis, the intermediate subset of monocytes has been described to expand. The non-classical subset is considered to have an important role in tissue repair due to its crawling behaviour on the endothelium, looking continuously for signs of inflammation or damage, in which case the cells can migrate rapidly in into the tissues. This capacity is enabled of the vastly expression of cytoskeletal genes involved in cell mobility. In other study,[22] which compares healthy volunteers with patients with chronic systolic Heart failure, an increased levels of the non-classical monocytes and reduced levels of the classical subset was described. Moreover, these changes were related to higher serum IL-13 levels and were inversely proportionated with increased size of the dysfunctional heart. In this study it was suggested that the non-classical subset may play an important role in the counterbalance mechanism of the organism, which aim to slow the remodelling process in the Heart failure patients.

Some data suggested that the non-classical subset could intervine in the resolving of the infection and the intermediate subset promotes it. A CD 16+ expansion has been observed also in bacterial and viral infections, but still it is not clear if they play a protective or host role in the organism in situation of infection.

In order to distinguish between the CD14+ and CD16+cells we normally use antibodies directed against the lipopolysaccharide-binding domain for the CD14+ and Fc-binding domain for the CD16+[44.]Same markers are also present in neutrophils and to exclude them it is necessary to use intracellular lactoferrin and CD66b to exclude granulocytes, CD56 to exclude natural killer

cells and staining for MHC class II expression and for low-level CD4 on monocytes can be useful[32]. However some other ways exists to analyse this population of cells, taking into consideration precisely the CD16+ cells, which, according to the group of investigators of Wong[35] could be based on the expression of Tie-2 and slan molecules. The Tie-2 molecule is present in up to 20% of all monocyte subsets, especially predominant, but not exclusive for the intermediate subset (also present in some other types of cells like circulating endothelial cell progenitors). Cells that express Tie-2 have proangiogenic activities, as promoting tumour angiogenesis and physiological wound healing processes. The slan+ cells, a new type of dendritic cells, are highly phagocytic cells, express CD16+ and have the capacity to stimulate CD8 T cells. Up to 30-50% of the non-classical monocytes are slan+. They have the ability to produce TNF alpha and pro-inflammatory nature.

2.3. Telomeres, definition and their clinical importance. Telomere length and attrition. The aging process and the telomeres

The telomere is a DNA-protein structure, a region of repetitive nucleotide sequence of the end of the chromosomes, which protects from deterioration of the DNA molecule and prevents from fusion events[48-50]. It comes from Greak: telos: end, and meros: part. In humans, the telomere is a DNA sequence of tract of tandem repeats of six-nucleotide unit TTAGGG single stranded overhang and is protected with a specific DNA protein, collectively called shelterin. The sheltering structure and sufficient telomere length are key elements for the protection of gene damage and genome destabilization. The telomere integrity is controlled by an enzyme called telomerase, which synthesizes anew the telomere sequences onto chromosome ends, but it cannot fully restore the telomere. During the cell division, in concrete, the chromosome replication, the duplication enzymes (DNA polymerase) cannot fully replicate the 3’ end of the

called chromated fibers, and, in order to protect them the telomere part of the telomere (between 30-150 base pairs) is lost in every cellular division. With the time, the telomere is progressively shortened, a phenomenon related to genetic instability, cellular senescence, pathogenesis and progression of a variety of human diseases[51]. Nevertheless, in case the cell enters in replicative senescence due to critical telomere length, it cannot continue with the division cycle and in this way a gene instability would be prevented as well as cancer. The enzyme telomerase is almost inactive in most somatic cells, and is up regulated in germ cells, some stem cells and in cancer cells.

The aging of the organism is a process of multiple physiological and structural changes on a molecular level, to which the human heart is not an exception. In every cell division the telomere is shortened reaching a critical length from which the cell cannot be replicated anymore. In order to protect gene modification and gene instability, in this moment the cell could become dysfunctional, enter in senesce (G0) or apoptosis and would be eliminated from the organism. The loss of the telomere DNA and its respective shortening could be accelerated from damaging environmental factors such as oxidative stress, metabolic endproducts, and ultraviolet radiation. For the optimal cellular division is also necessary the integrity of the shelterin protein, the telomere DNA and the telomere tridimensional loop structure[48-51]. Moreover, when some of these elements are altered or damaged, it can induce chromosomal instability and cell death. Consequently, the telomere attrition and respectively the telomere length have been considered parameters for aging.

Telomere length varies extremely between individuals at the same age, starting from birth and it has a heritability component (up to 82%).[51]

2.4. Telomere and cardiovascular diseases.

From previous studies we know that patients with chronic heart failure tend to have also shorter telomeres as compared to healthy individuals[52]. Many predisposing factors for heart failure (arterial hypertension, arteriosclerosis, diabetes, smoking, myocardial infarction[53-55] independently have a negative effect on the shortening of the telomere. However, it is not very clear if the progression of the disease itself produces the shortening of the telomere or the telomere attrition is the cause of progression of the heart failure. Moreover, the telomere length has been associated with the severity of symptoms and outcome in chronic heart failure.[52,56] Other authors have described that accelerated senescence due to short telomere length could facilitate and even cause heart failure[57]. Nevertheless, oxidative stress and increase inflammatory condition, presented in heart failure, can also predispose to telomere attrition and facilitate the development of heart failure.

Previous studies have shown that mice with very short telomeres developed left ventricular failure and pathological cardiac remodelling, similar to end stage of dilated cardiomyopathy in humans. Moreover, some studies have suggested that there is accelerated telomere shortening in cardiovascular diseases[58-62], can not only be a normal process of aging but a pathological mechanism of the disease. When hearts from patients with dilated cardiomyopathies were compared to hearts without disease, the myocytes of the diseased hearts had significant telomere shortening and cell death, and shorter telomeres have been associated with worse outcomes and higher NYHA class; indeed, ischaemic aetiology was an additional factor which correlated with shorter telomeres in this clinical scenario. The patients with renal insufficiency and reduced ejection fraction have also shorter telomere length compared to those without[59,63,64].

Different diseases and habits have been associated with reduced telomere length as type I and II diabetes as well as insulin resistance[65,66], arterial hypertension[67,68], atherosclerosis and

myocardial infarction, smoking and cumulative dose pack-years[52,69,70-72]. The exact pathophysiology is not clear but it is believed that smoking promotes oxidative stress, lessens vasomotor function of the vessels and increases inflammation and platelet activation.

2.5. Telomere length

The telomere length is an inheritable clinical parameter, since it is directly related with the cell division and the telomere attrition, which are markers for cell aging due to genetic instability, pathogenesis and progression of different kind of diseases. Since each cell has many telomeres and every telomere has its own length, the complete length is the mean telomere length and the length distribution for a given cell type.

Nowadays, several established techniques to assess telomere length such as Southern blot, PCR, single telomere length analysis and fluorescence in situ hybridization coupled with flow cytometry (flow-FISH), restriction fragment analysis exist. Many of these analyses are time consuming as well as expensive and require large amounts of sample and specific antibodies conjugated to heat-stable fluorochromes with limited sensitivity and imprecisely measurements.

3. HYPOTHESIS

3.1. Hypothesis of the project

The distribution and prognostic significance of the different monocyte subsets and their telomeres could be an independent predictor factor for cardiovascular events and, in concrete, for heart failure related hospitalizations and mortality in patients with heart failure.

If this hypothesis turn out to be true, in the future, the monocytes and their telomeres could be used not only as a predictor factor but also might be taken into consideration as part of an immuno-modulation therapy in the future for the heart failure patients.

3.2. Justification of the project

Heart failure is a syndromic disease with high prevalence in the developed countries and carries elevated morbidity and mortality and high economic burden. A better understanding of the disease, its pathophysiology and progression, as well as a more precise stratification of the patients with established prognostic factors is of crucial importance for an optimal treatment of heart failure. Frequently used prognostic factors as New York Heart Association (NYHA) functional classification, echocardiographic parameters (the left ventricle ejection fraction (LVEF)), and anthropometric parameters as age, sex, aetiology, comorbidities and laboratory markers (NT-proBNP, BNP, Na, etc.) do not allow to completely and individually predict disease progression and mortality, although in the last years, the search for biomarkers and new techniques for classification and treatment has generated a vast field of investigation in heart failure[10].

New prognostic factors have to be discovered and apply, which might help to better assess heart failure prognosis at an individual level and more accurately stratify the risk of heart failure patients.

Little is known about the significance and importance of the monocyte count and their distribution in heart failure, especially the role that these can play as a predictor factor for the severity and progression of the disease[45]. Previous studies have describe the importance of the monocytes in other cardiovascular events[22,73-75] as in other autoimmune conditions, viral and infectious diseases and other human diseases[35]. In this context of searching for better improvement of risk stratification in heart failure patients we studied the distribution and its prognostic significance of the monocytes in heart failure patients from the Heart Failure Unit.

4. OBJECTIVES OF THE PROJECT

4.1. Main objective

To study the distribution and significance of the circulation monocytes in heart failure and their possible relationship with mortality and heart failure related hospitalization of patients with heart failure from the Unit of Heart Failure at our Hospital during a follow-up of at least one year.

Elena Elchinova, Iris Teubel, Santiago Roura, Marco A. Fernandez, Josep Lupón, Carolina Galvez Montón, Marta de Antonio, Pedro Moliner, Mar Domingo, Elisabet Zamora, Julio Núñez, Germán Cediel, Antoni Bayes-Genıs. Circulating monocyte subsets and heart failure prognosis. PLoS One. 2018 Sep 21;13(9):e0204074. doi: 10.1371/journal.pone.0204074. eCollection 2018[76].

4.2. Secondary objectives

1. To develop a new, more precise and cheaper method for assessing the telomere length in the monocytes and at the same time performing monocyte subset classification, using a FISH-flow technique. From previous investigations[77-79] we know that this process could be difficult, expensive and time consuming, with uncertain success.

Santiago Roura, Marco A Fernández, **Elena Elchinova**, Iris Teubel, Gerard Requena, Roser Cabanes, Josep Lupón and Antoni Bayes-Genis. Brilliant violet fluorochromes in simultaneous multicolor flow cytometry–fluorescence in situ hybridization measurement of monocyte subsets and telomere length in heart failure. Lab Invest. 2016 Nov;96(11):1223-1230. doi: 10.1038/labinvest.2016.100. Epub 2016 Sep 12.[80]

2. To investigate a particular part of the monocytes: their telomeres; due to their unique characteristics, the telomeres are associated with the stabilization and protection of the DNA molecule during the cellular division. Telomeres and, in concrete, the enzyme telomerase,

have been the focus of many previous studies in the medical field. It has already demonstrated their key role in processes such as cancer and aging. We specifically aimed to address the telomere length, an important parameter, related to the stability of the telomere. As far as we were concern, there was not any study analysing the telomere length and its relevance in monocytes of heart failure patients, even less the possibility to be considered a prognostic factor in this group of patients.

Iris Teubel, **Elena Elchinova**, Santiago Roura, Marco A. Fernández, Carolina Gálvez-Montón, Pedro Moliner, Marta de Antonio, Josep Lupón and Antoni Bayés-Genís. Telomere attrition in heart failure: a flow-FISH longitudinal analysis of circulating monocytes. J Transl Med. 2018 Feb 20;16(1):35. doi: 10.1186/s12967-018-1412-z. PMID: 29463269.[81]

5. PUBLISHED STUDIES

5.1. First Study

Title:

Brilliant violet fluorochromes in simultaneous multicolor flow cytometry–fluorescence in situ hybridization measurement of monocyte subsets and telomere length in heart failure.

Authors:

Santiago Roura*, Marco A Fernández*, **Elena Elchinova***, Iris Teubel, Gerard Requena, Roser Cabanes, Josep Lupón and Antoni Bayes-Genis.

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Brilliant violet fluorochromes in simultaneous multicolor flow cytometry–fluorescence *in situ* hybridization measurement of monocyte subsets and telomere length in heart failure

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Conventional analytical methods to determine telomere length (TL) have been replaced by more precise and reproducible procedures, such as fluorescence *in situ* hybridization coupled with flow cytometry (flow–FISH). However, simultaneous measurement of TL and cell phenotype remains difficult. Relatively expensive and time-consuming cell-sorting purification is needed to counteract the loss, due to stringent FISH conditions, of prehybridization fluorescence by the organic fluorochromes conventionally used in the phenotyping step. Here, we sought to assess whether the newly developed Brilliant Violet (BV) dyes are valuable to specifically and simultaneously assess the distribution and telomere attrition of monocyte subsets circulating in the blood of a cohort of patients with heart failure. We performed flow–FISH on blood samples from 28 patients with heart failure. To differentiate among monocyte subsets, we used BV and conventional fluorochromes conjugated to antibodies against CD86, CD14, CD16, and CD15. We simultaneously assessed the TLs of the monocyte subsets with a telomere-specific peptide nucleic acid probe labeled with fluorescein isothiocyanate. The BV dyes completely tolerated the harsh conditions required for adequate DNA denaturation and simultaneously provided accurate identification of monocyte subpopulations and respective TLs. The presented protocol may be faster and less expensive than those used currently for purposes such as establishing associations among patient categories, disease progression, monocyte heterogeneity, and aging in the context of heart failure.
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Determination of telomere length (TL) is clinically valuable, since it reflects both the number of times the cell has divided and telomere attrition; both of these attributes are associated with the genetic instability, pathogenesis, and progression of a variety of human disorders. Current laboratory techniques for TL measurement include Southern blot, PCR, single TL analysis, and fluorescence *in situ* hybridization coupled with flow cytometry (flow–FISH). However, these methodologies have critical limitations; for example, they require large sample amounts and antibodies that are preferably conjugated to heat-stable fluorochromes, their analytical sensitivity is suboptimal when very short telomeres are assessed, and measurements among different samples vary excessively.[1]

Flow–FISH is a methodology that has been thoroughly validated for clinical diagnosis, and it includes the option to analyze expression of specific surface antigens in either single cells or cell subsets.[2,3] However, previous studies have revealed that combining cell phenotyping by flow cytometry with TL by FISH is difficult due to the harsh conditions required for hybridization of the telomere probe (ie, intense fixation and incubation at 82 °C). Relatively expensive and time-consuming cell subset purification (eg, by cell-sorting) is required to counteract the loss of fluorescence of the conventional, non-heat-stable, organic fluorochromes that are usually used in the immunophenotyping step. As an alternative, a small group of fluorochromes, including

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Quantum Dots (QD), cyanines, and Alexa Fluor dyes, have been reported to partially resist these stringent experimental conditions.[4,5] However, it is difficult to conjugate QD with antibodies and therefore their use in detecting cell surface antigens is limited. To that end, researchers must usually isolate the cell subset of interest prior to performing flow–FISH.

The Brilliant Violet (BV) family of fluorochromes has unique optical properties to produce much brighter fluorescence signal than most of the traditional fluorescent dyes used in flow cytometry. Based on chemistry awarded with Nobel Prize, they are generated by about 1000 fluorescent monomeric subunits per macromolecule that act cooperatively along the entire length of the polymer backbone, resulting in emission of light with high molecular extinction coefficients and high quantum efficiency.[6,7] Other advantages of this new class of dyes include synthetic modifications of their backbone structure to span the full range of the visible spectrum, and spectral compatibility with each other for multicolor simultaneous labeling.[8]

Heart failure remains a growing medical problem related to major hospitalization, mortality and poor prognosis worldwide.[9] Although advances in diagnosis and treatment over last decades, many of the pathophysiological mechanisms and prognostic markers remain poorly investigated. In this context, the assessment of monocyte heterogeneity and/or ageing as potentially valuable predicting factors of categories and progression of heart failure is challenging. To that end, standardized instrument settings, reagents, and sample preparation and analytical protocols are required.

Thus, in the present study, we used the newly developed BV fluorochromes to specifically and simultaneously measure the distribution of circulating monocyte subsets and their respective TLs (RTLs) in a cohort of patients with heart failure.

MATERIALS AND METHODS

Study Population

We enrolled 28 consecutive patients diagnosed with heart failure and collected 3 ml of blood into EDTA tubes by conventional forearm venipuncture. The main clinical and demographic characteristics of the patients are summarized in Table 1. The study protocol was approved by the Clinical Research Ethics Committee of our institution, and it conformed to the principles outlined in the Declaration of Helsinki. Written informed consent was previously obtained from each subject. Samples were stored at room temperature and processed within 4 h after collection.

Table 1 Demographic and clinical characteristics of patients with heart failure

	N = 28
Age	64.9 ± 12
Male	20 (71,4%)
Etiology	
Ischemic	12 (42.9%)
IDCM	6 (21.4%)
Hypertensive	1 (3.6%)
Enolic	1 (3.6%)
Toxic	1 (3.6%)
Valvular	4 (14.3%)
Hypertrophic	2 (7.2%)
Others	1 (3.6%)
Duration heart failure (months)	28 (11–72)
LVEF	40 ± 12
NYHA classification	
I–II	22 (78,6%)
III–IV	6 (21.4%)
Diabetes mellitus	9 (32.1%)
Hypertension	14 (50%)
Previous myocard infarction	9 (32.1%)
Treatment	
ACEI/ARBII	25 (89.3%)
Beta-blockers	26 (92.9%)
MRA	17 (60.7%)
Loop diuretics	15 (53.6%)
Digoxin	8 (28.6%)
Ivabradine	8 (28.6%)
CRT	3 (10.7%)
ICD	4 (14.3%)

Abbrevations: ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CRT, cardiac resynchronization therapy; ICD, implantable cardioverter-defibrillator; IDCM, idiopathic dilated cardiomyopathy; LVEF, left ventricular ejection fraction; MRA, mineralocorticoid receptor antagonists; NYHA, New York Heart Association.

Multicolor Flow–FISH Procedure

Immunophenotyping

Whole-blood samples were lysed by incubating them with PharmLyse solution (BD Bioscience, San Diego, CA, USA) for 10 min. They were then washed twice with phosphate-buffered saline (PBS; Gibco Life Technologies/Invitrogen, Carlsbad, CA, USA) containing 0.5% bovine serum albumin (Miltenyi Biotech, Bergisch Gladbach, Germany). Cell concentration was determined by flow cytometry using Perfect-Count beads (Cytognos, Salamanca, Spain). We stained 1×10^6 cells with titrated amounts of antibodies conjugated to BV or Alexa Fluor dyes and specific to human CD86-BV605, CD14-BV785 (Biolegend, San Diego, CA, USA), CD16-BV421 and CD15-AlexaFluor647 (BD Biosciences) in 50 μl of Brilliant Stain Buffer (BD Biosciences) for 15 min at room temperature (RT). We also comparatively tested a battery of commercially available antibodies against human

Table 2 BD Fortessa SORP optical configuration

Splitter	BP filter	Δ AutoFL
Violet laser (405 nm)		
750 LP	780/60	Low
670 LP	710/50	Medium
630 LP	660/20	High
595LP	605/40	High
550 LP	585/42	High
535 LP	560/40	High
505 LP	515/20	High
None	450/50	High
Blue laser (488 nm)		
750 LP	780/60	Medium
670 LP	705/70	Medium
635 LP	670/14	High
600 LP	616/23	High
None	575/26	High
Green laser (532 nm)		
750 LP	780/60	Medium
670 LP	705/70	Medium
635 LP	670/14	High
600 LP	616/23	High
None	575/26	High
Red laser (640 nm)		
735 LP	780/60	Low
690 LP	710/50	Low
None	660/20	Low

All fluorescence channels are named with the first letter of the laser (B, V, G, and R) and the central wavelength of the band-pass filter. Mirrors-spliters and band-pass filters are shown. Last column lists the level of increase in cellular autofluorescence in FISH protocol: High (>5-fold), Medium (2- to 5-fold) and Low (<2-fold) regarding background (non-FISH protocol) level.

CD8 coupled to BV421, BV510, BV570, BV605, BV650, BV711, or BV786 (Biolegend; BD Biosciences) or PE, PECy7, APC, PerCP, or BB515 (BD Biosciences) bound to compensation capture beads (OneComp eBeads; eBioscience, San Diego, CA, USA). After a brief centrifugation and two washes in PBS, cells were fixed with 6 mM bis sulfosuccinumidyl suberate (Sigma-Aldrich) for 30 min at 2–8 °C. The reaction was then immediately quenched by incubating with 1 M Tris buffer (pH 8.0) for 15 min at RT, and residual red blood cells were removed using FACS lysing solution (BD Biosciences) for 7 min at RT.

In situ hybridization

FISH was performed with the Telomere PNA kit (Dako, Glostrup, Denmark) in accordance with the manufacturer's instructions and using the lymphoblastic leukemia 1301 cell line as an internal control. An equal volume (300 μl) of hybridization solution with or without FITC-labeled PNA probe (telomere-specific peptide nucleic acid probe) was added to sample and control tubes, respectively. Tubes were then hybridized for 10 min at 82 °C in a dry block heater (Grant Instruments, Cambridge, UK), and thereafter incubated overnight at RT. Tubes were washed twice in wash solution with previous incubation at 40 °C, and resuspended in 500 μl of DNA-staining solution for 2–3 h at 2–8 °C. Samples were acquired by flow cytometry, and a minimum of 10 000 monocytes were collected in each sample tube. Correction for DNA ploidy of the blood sample *versus* the internal control was performed as described previously.[10]

Flow cytometry analysis

All samples were acquired on a Fortessa SORP flow cytometer (BD Biosciences) using 20 parameters (two scattering and 18 fluorescence detectors). The cytometer was equipped with four lasers with the following configurations: 100-mW 488 nm, 150 mW 532 nm, 50 mW 405 nm, and 100 mW 640 nm. All fluorescence channels were referred to by the first letter of the laser (B, V, G, and R), and the central wavelength of the band-pass filter (summarized in Table 2). We performed routine daily quality control tests with Cytometer Setup & Tracking Beads (BD Biosciences) in accordance with the manufacturer's instructions. The initial optimal voltage ranges and linearity for photomultipliers were selected using 6-peak Rainbow Calibration Particles and Unstained Comp-Beads (BD Biosciences), as described previously.[11] Owing to the increased cellular autofluorescence associated with FISH, PMT voltages were readjusted to obtain maximum sensitivity and resolution. A compensation matrix was applied by single-fluorochrome controls processed with the flow-FISH protocol. Data were analyzed with FACSDiva (BD Biosciences) and FlowJo (Treestar, Ashland, OR, USA) software.

We initially gated for G0/G1 cells of both leukocyte subsets and 1301 cells based on DNA content and then by scatter properties. Neutrophils and monocytes had similar scatter patterns after FISH; thus, monocytes were sequentially selected based on a CD86 *versus* CD16 plot, and a CD15 *versus* CD16 plot to gate out neutrophils. The gated monocytes were then analyzed for CD14 and CD16 expression. Clumped cells were excluded by propidium iodide (PI) area *versus* PI width plot. Each subset of monocytes and internal control cells was subsequently displayed on a plot comprising the FITC-labeled PNA probe on PI (B695-A) *versus* the PNA probe (B515-A), and the median fluorescence intensity (MFI) of the PNA probe was calculated. We determined the gate boundaries for CD14- and CD16-positive subsets with the appropriate fluorescence minus one controls. For bead-based comparative purposes, beads

were gated in forward and side scatter plots, and the MFIs of both negative and positive fluorochrome capture beads were analyzed.

The RTL value for each monocyte subset was calculated as the ratio between the MFI of each subset and the MFI of the control cells. Corrections were made for the DNA index of G0/G1 cells, as described previously.[12]

RESULTS

In these experiments, we assessed whether the newly developed BV fluorochromes were useful for flow–FISH. To that end, we attempted to simultaneously determine the identification of the three mononuclear cell subsets referred to as classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$) and nonclassical ($CD14^{low}CD16^+$), as well as their respective RTLs in a cohort of patients with heart failure.

We first tested the thermal stability of BV dyes at 82 °C and the effects of the extensive fixation step that takes place in standard FISH protocols. We did so by analyzing the fluorescence emissions of different commercially available BV fluorochromes coupled to antibody capture beads. This allowed us not only to exclude the increased cellular autofluorescence that occurs during FISH but also to avoid changes in the antigen–antibody complex that can be provoked by the harsh conditions associated with FISH. The polymer-based BV dyes were heat stable and withstood the stringent conditions of hybridization with no degradation or loss of emission (Figures 1a–g). Additionally, the recently developed polymer-derived fluorochrome Brilliant Blue 515 showed the same thermal resistance (Figure 1h). By contrast, conventional organic or protein-based fluorochromes such as phycoerythrin, allophycocyanin, and peridinin–chlorophyll protein and related tandem fluorochromes were commonly completely degraded and their emissions abrogated during FISH (Figures 1I and k).

To determine if the stability of the BVs was sustained in a cell-based protocol, we incubated whole-blood leukocytes extracted from patients with titrated amounts of anti-human CD8-BV421, CD14-BV786, CD15-Alexa647, CD16-BV421, and CD86-BV605, and assessed them by standard FISH procedures. Although several of the photomultipliers that we evaluated in the violet laser line showed an increase from medium to high levels of cellular autofluorescence after fixation and incubation at 82 °C (most pronounced between wavelengths 515 and 660 nm; Table 2), discrimination among $CD8^+$, $CD14^+$, $CD15^+$, $CD16^+$, and $CD86^+$ cell subsets related to the nonhybridization protocol was preserved (Figure 2). Table 2 shows the increased cellular autofluorescence associated with the FISH protocol. Increases in cellular autofluorescence were classified as High (>5-fold), Medium (2- to 5-fold), or Low (<2-fold) with respect to background levels. All of the BV dyes retained >60% of their pre-FISH Stain Index (data not shown).

We next assessed whether using BV-conjugated antibodies would allow us to discriminate among the different monocyte subsets circulating in patients' blood by measuring cell surface expression of CD14 and CD16. We found that the BV-conjugated antibodies successfully discriminated among the monocyte subsets (Figure 3). Moreover, percentages of monocyte subsets measured before (after stopping BS3 crosslinking with Tris buffer) and after hybridization were very similar when measured with CD15-AlexaFluor647, CD16-BV421, CD86-BV605, and CD14-BV785, all of which are spectrally compatible with FITC-labeled PNA and PI (Figure 3). The calculated RLTs corresponding to each monocyte subset were 9.68 ± 2.21, 9.76 ± 2.26, and 9.96 ± 2.20 for classical, intermediate, and nonclassical monocytes, respectively (Figure 4).

DISCUSSION

In the present study, we evaluated whether BVs, a novel family of polymer-based fluorescent dyes, are valuable as heat-stable alternatives to conventional organic or protein-based fluorochromes in flow–FISH. We used the BVs to simultaneously measure the different circulating monocyte subpopulations and their respective TLs in blood samples from a cohort of patients with heart failure.

In general, current flow–FISH measurement of TL requires targeted cell purification (eg, by cell sorting).[13] As an alternative, some studies have attempted to identify cell subsets by immunophenotyping and then applying TL calculations to all cells in a heterogeneous sample such as venous blood.[14] Other studies have used a variety of heat-stable fluorochromes.[15] In protocols in which targeted cells must be isolated prior to flow–FISH measurement of TL, it is also difficult to obtain the necessary number of cells for TL. Other limitations include thermal instability of conventional fluorochromes and high levels of cellular autofluorescence during the analytical process.[16] Although other fluorochromes such as QD, cyanines, and Alexa Fluor are relatively thermally stable under the harsh conditions required for hybridization, their implementation is limited by the scarcity of appropriate reagents, the difficulty associated with conjugating them to antibodies, their excitation by several wavelengths of lasers,[17] and the overlap of cellular autofluorescence and dye emissions.

Their polymeric structure and their benefit in multicolor staining panels suggest that the BV family of dyes may be successful alternatives to the brightest fluorochromes commonly used for immunophenotyping purposes, such as phycoerythrin and allophycocyanin. Moreover, their use enables the violet laser for polychromatic excitations. Additional major advantages of BV-based flow cytometry include their high solubility; lack of nonspecific binding; compatibility with intracellular staining; and efficient, stable conjugation to antibodies.[6] BV dyes are more thermally stable than most low molecular weight fluorochromes, which lose their fluorescence under the intense fixation and high temperature (82 °C) required for DNA denaturation and hybridization of the fluorescent telomere probe.

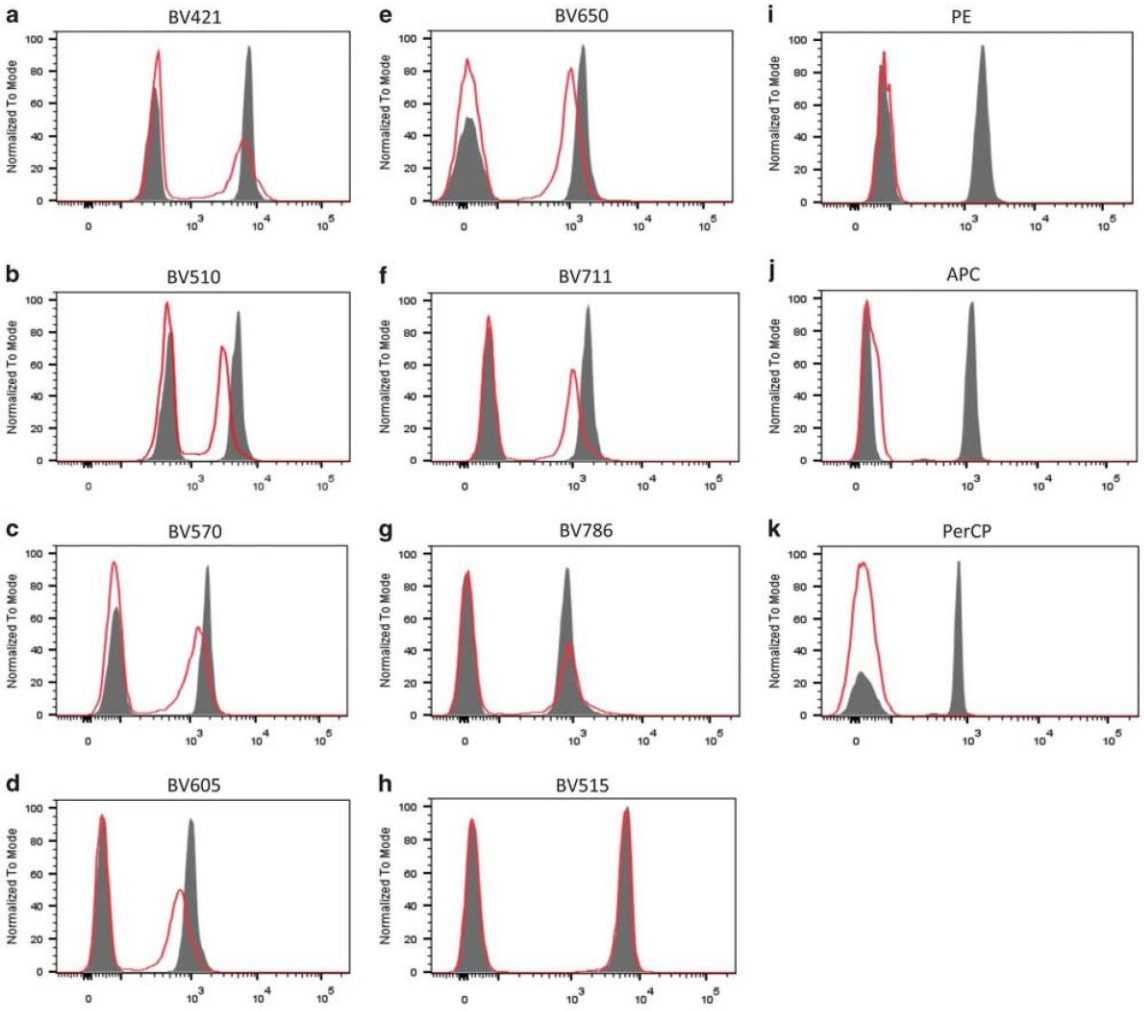

Figure 1 Direct effect of FISH protocol on Brilliant Violet fluorochromes. Detection of BV421 (**a**), BV510 (**b**), BV570 (**c**), BV605 (**d**), BV650 (**e**), BV711 (**f**), BV786 (**g**) and BB515 (**h**) fluorescence signal using dye-labeled (right peak) and unlabeled (left peak) beads. Each BV signal is compared in FISH (red line histograms) and in non-FISH conditions (gray filled histograms). In contrast, conventional organic fluorochromes such as phycoerythrin (**i**), allophycocyanin (**j**) and peridinin chlorophyll (**k**) suffer complete degradation and subsequent emission abrogation under FISH conditions.

Based on the qualities described above, we hypothesized that BVs, as well as the new polymer-based dye Brilliant Blue BB515, would be appropriate alternatives to conventional fluorochromes in assays designed to simultaneously identify specific cell subpopulations (immunophenotyping) and hybridize probes to telomeres *in situ*. We also tested stabilization of the fluorochrome–antibody complex with the amine-to-amine cross-linker bis sulfosuccinumidyl suberate. This cross-linker is homobifunctional, water-soluble, noncleavable, and membrane impermeable; all of these properties are essential for BV-derived prehybridization fluorescence emission.[18]

To evaluate which BV dyes perform best in the detection of CD14, CD16, and CD86 expression in monocyte subsets and TL calculation, we measured the levels of cellular autofluorescence induced by treatment and the spectral overlap of DNA dyes required for the identification of cell cycle phases. In our hands, PI staining decreases sensitivity in V560–V660 channels (data not shown), and this was taken into account. Moreover, fluorochrome selection must be optimal relative to expected antigen densities and dye efficiencies.[19] Thus, in the present study, CD16-BV421, CD86-BV605, and CD14-BV786 were chosen for scrutiny.

Monocyte subsets play an important role in inflammation and innate and adapted immunity, and their contribution to cardiovascular diseases is currently being researched.[20–23] In this context, our aim was to identify alternative fluorochromes for use in multicolor flow–FISH to differentiate

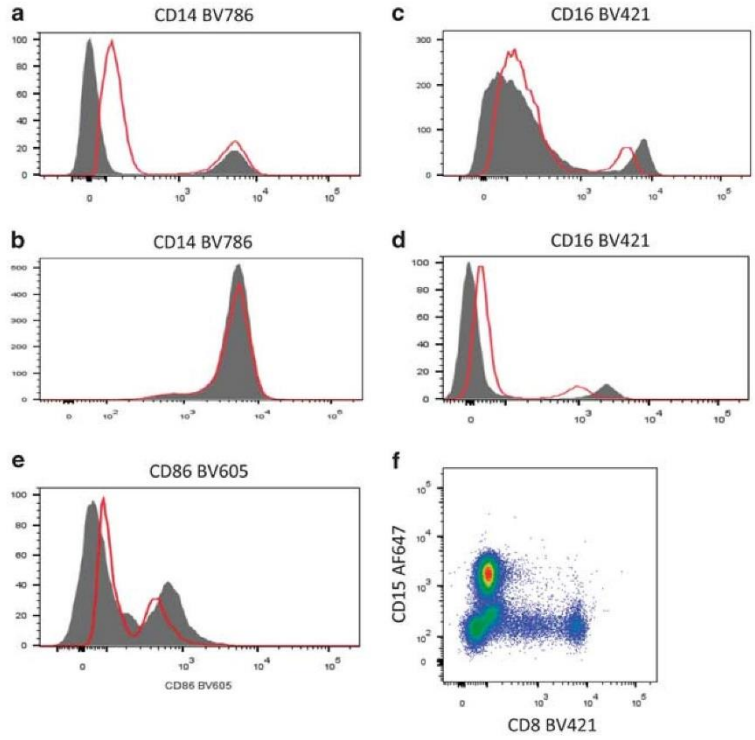

Figure 2 Comparative nonhybridization (gray filled histograms) and hybridization (red line histograms) staining protocols. Peripheral blood mononuclear cells (**a**) and monocytes (**b**) stained with CD14-BV786. Although increased cellular autofluorescence is shown using the FISH protocol (a), $CD14^+$ cell staining is unaffected (**a**, **b**). Monocytes (**c**) and lymphocytes (**d**) stained with CD16-BV421. Both cell populations can be clearly discriminated from background. Peripheral blood mononuclear cells stained with CD86-BV605 (**e**). FISH protocol in a whole-blood sample using heat-stable BV421 and Alexa647-conjugated antibodies for specific detection of $CD8^+$ lymphocytes and $CD15^+$ neutrophils (**f**).

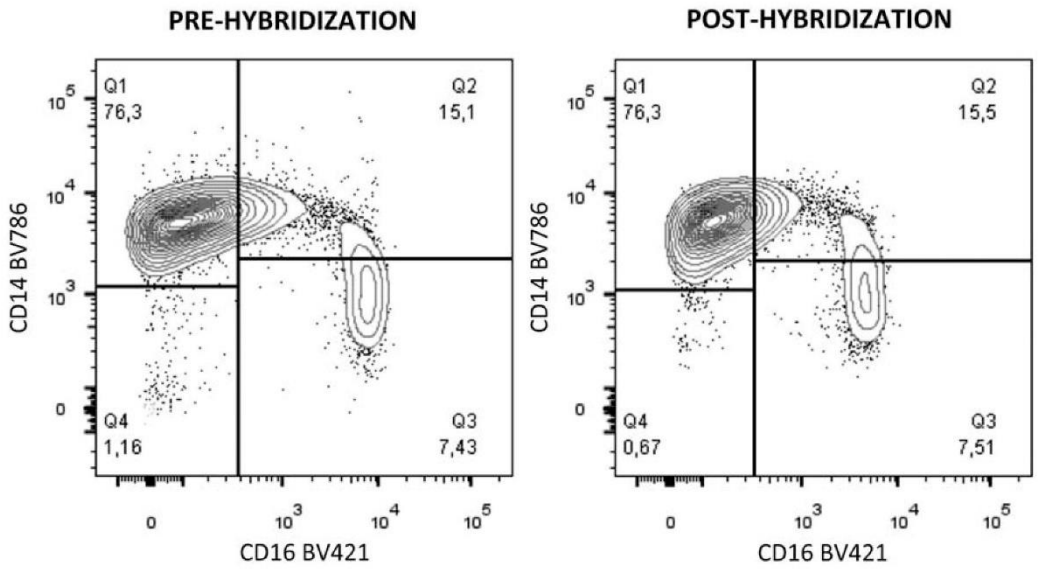

Figure 3 Representative analysis of whole-blood sample from a patient with heart failure. Monocyte subsets showing different expression levels of CD14 and CD16 cell surface antigens under prehybridization (left contour plot) and post-hybridization (right contour plot) conditions. Both plots depict a similar fluorescence intensity and frequency pattern of the different monocyte subsets.

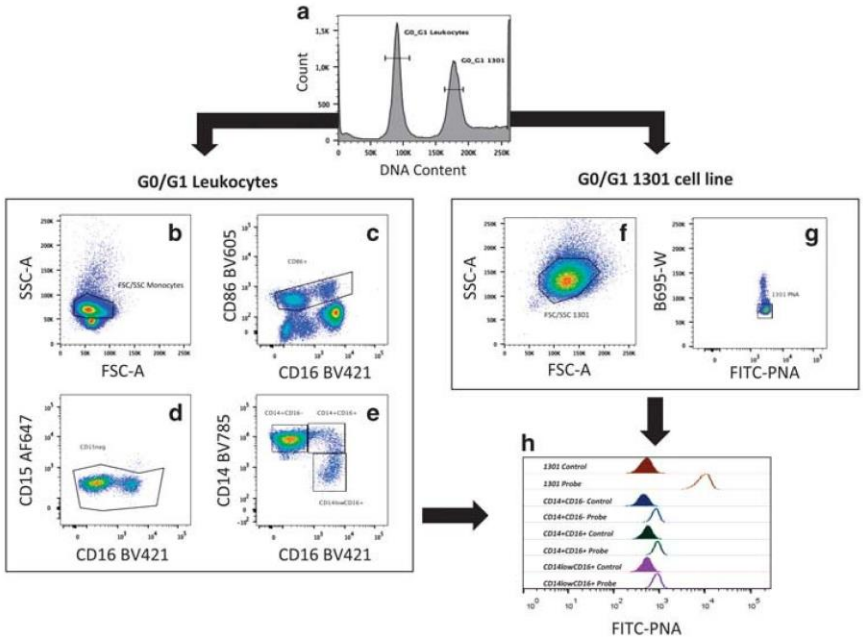

Figure 4 Gating strategy for the RTL analysis in monocyte subsets. G0/G1 cells were selected in DNA content histogram (**a**) and then gated by scatter properties (**b**, monocytes and **f**, 1301 cell line). CD86 *versus* CD16 (**c**) and CD15 *versus* CD16 (**d**) plots were used to specifically select monocytes; thereafter both CD14 and CD16 expression were analyzed (**e**). (**h**) Hybridization of FITC-labeled PNA probe and control (without probe) in monocyte subsets based on the CD4 and CD16 expression and in 1301 cell line, which was used as internal reference control (**g**).

among monocyte subpopulations and quantify their RTLs. Moreover, fixation steps can lead to loss of differentiation based on scatter properties, and monocyte subsets expressing CD14 and CD16 antigens are not easily separated from other $CD16^+$ leukocytes such as neutrophils. To address this concern, we confirmed that discrimination between monocytes and neutrophils requires the employment of additional cell surface antigens, such as an exclusion marker for neutrophils (eg, CD15).

Taken together, our results show that BVs used in flow–FISH can facilitate accurate, specific, relatively inexpensive (approximately 3-fold less expensive), and faster measurement of circulating monocyte subsets and their RTLs in the context of heart failure. Therefore, our technical protocol may be used to further explore important associations among patient categories, disease progression, monocyte heterogeneity, and aging in series that comprise broader ranges of patients than those assessed in conventional studies.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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5.2. Second Study

Title:

Circulating monocyte subsets and heart failure prognosis.

Authors:

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RESEARCH ARTICLE

Circulating monocyte subsets and heart failure prognosis

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Abstract

Monocytes are a heterogeneous population of effector cells with key roles in tissue integrity restoration and maintenance. Here, we explore the association of monocyte subsets and prognosis in patients with ambulatory heart failure (HF). Monocyte subsets were classified as classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{++}/CD16^{+}$), or non-classical ($CD14^{+}/CD16^{++}$). Percentage distribution and absolute cell count were assessed in each subset, and multivariable Cox regression analyses were performed with all-cause death, HF-related hospitalization, and the composite end-point of both as dependent variables. 400 patients were consecutively included (72.8% male, age 69.4±12.2 years, 45.5% from ischemic aetiology, left ventricle ejection fraction (LVEF) 41.6% ±14.5, New York Heart Association (NYHA) class II 62.8% and III 30.8%). During a mean follow-up of 2.6±0.9 years, 107 patients died, 99 had a HF-related hospitalization and 160 suffered the composite end-point of all-cause death or HF-related hospitalization. Monocyte subsets assessed in percentages were not independently associated to any of the end-points. When considering number of cells/μL, intermediate subset was independently associated with an increase of all-cause death (HR 1.25 [95% CI 1,02–1.52], p = 0.03), and the composite end-point HR 1.20 [95% CI 1,03–1.40], p = 0.02). The presented findings show that absolute cell count of monocyte subsets was preferred over monocyte percentage for prognosis stratification for outpatients with HF. The intermediate monocyte subset provides information on increased risk of all-cause death and the composite end-point.

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Introduction

Heart failure (HF) is a syndromic disease associated with significant economic burden and clinical manifestations [1,2]. Morbidity and mortality continues to be unacceptably high for patients with HF, despite significant research and medical progress. A better risk stratification by an improved understanding of the underlying pathogenic mechanisms and potentially valuable prognostic markers could be a key for the optimal determination of patients who would benefit from close follow-up and more aggressive treatment. However, established prognostic factors including the New York Heart Association (NYHA) functional classification, the left ventricle ejection fraction (LVEF), age, sex, aetiology, comorbidities and laboratory markers all fail to completely and individually predict disease progression and mortality [3–6]. Risk stratification could be improved by the incorporation of biomarkers associated with different pathophysiological pathways, not reflected by established mortality risk factors. The search for biomarkers for HF diagnosis and prognosis has become a major research focus over the last decade [6–8].

The significance and importance of monocyte count and subset distribution in HF is unknown, and their ability to act as prediction factors for the severity and progression of the disease is not yet well established [9,10], although previous reports have indicated an association between monocyte subpopulations and cardiovascular events in different pathologies [11–14]. Monocytes and their macrophage derivates play a crucial role in the immune system, and participate in host defense, immunoregulation, and tissue repair. In particular, there are three distinct subsets of monocytes, defined as classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{++}/CD16^{+}$) or non-classical ($CD14^{+}/CD16^{++}$) by Ziegler-Heitbrock and colleagues among others [15], which can be differentially detected by multicolor flow cytometry analysis [16].

In the present study, we investigate the clinical relevance of monocyte count and distribution in patients with ambulatory HF, and the value of monocyte subsets to establish disease prognosis.

Materials and methods

Study population

Ambulatory patients consecutively treated at a multidisciplinary HF Clinic from December 2013 to April 2015 were included in this study, independently of the data of their entry into the HF Clinic program. The referral inclusion criteria have been described elsewhere [17,18]. All study procedures were performed in accordance with the ethical standards outlined in the Helsinki Declaration of 1975 as revised in 2013 [19], this study was approved by the local ethics committee (Comitè d'Ètica de la Investigació, Hospital Universitari Germans Trias i Pujol, Ref. CEI: PI-13-057), and all participants provided written informed consent.

Follow-up and outcomes

All patients made follow-up visits at regular, predefined intervals, and made additional visits as required in cases of decompensation [17,18]. The schedule included a minimum of quarterly visits with nurses, biannual visits with physicians, and elective visits with geriatricians, psychiatrists, nephrologists, and rehabilitation physicians. Patients were contacted by telephone if they did not attend a regular visit.

The primary end-points were all-cause death and the composite of all-cause death or HF-related hospitalization. Fatal events were identified from electronic clinical records and by contact with the patients' relatives if necessary. When needed, data were verified by

comparison with records stored in the databases of the Catalan and Spanish health systems. Events were adjudicated by two of the authors (EE and JL) and three clinical and research nurses (BG, JG and JR). Follow-up was closed in May 2017.

Blood extraction and flow cytometry analysis

Peripheral blood samples (~3 mL) were collected into EDTA tubes via standard forearm venipuncture performed between 9:00 am and 11:00 am, and processed within 4 h post-collection. Samples and data from patients included in this study were processed and collected by the IGTP-HUGTP Biobank integrated in the Spanish National Biobanks Network of Instituto de Salud Carlos III (PT13/0010/0009) and Tumour Bank Network of Catalonia.

In brief, 100 μL of fresh peripheral blood (tube A) was stained with titrated amounts of anti-human CD86-BV605, CD14-BV785, and CD16-BV421 antibodies (Biolegend, San Diego, CA) during 15 min in the dark at room temperature. Red blood cells were then lysed following 10-minute incubation with 2 mL PharmLyse solution (BD Biosciences, San Diego, CA), and the resulting cell suspension was washed twice with phosphate-buffered saline. For monocyte absolute cell counts, 50 μL of unprocessed blood from the same donor were also stained (tube B) with anti-human CD86-PE (BD Biosciences) antibody and 50 μL Perfect-Count beads (concentration ranging from 1,056 to 1,067 beads/μL, depending on batch; Cytognos, Salamanca, Spain) were also added as reference counting beads in a lyse no-wash method. Fluorescence minus one controls were used to determine positive and negative staining boundaries for CD14 and CD16 antigens.

Monocyte subsets were classified as $CD14^{++}/CD16^{-}$ (classical), $CD14^{++}/CD16^{+}$ (intermediate), and $CD14^{+}/CD16^{++}$ (non-classical). Frequency of each monocyte subset regarding total monocyte population ($CD86^{+}$) were obtained from tube A and absolute counts (expressed as number of cells/μL) were calculated by multiplying the percentage of each subset by the total monocyte population ($CD86^{+}$) count performed from tube B. All samples were acquired in the next hour after staining protocol on a Fortessa SORP flow cytometer (BD Biosciences) equipped with four lasers (100-mW 488 nm, 150mW 532 nm, 50mW 405 nm, and 100mW 640 nm) by using the sample acquisition and analysis FACSDiva v6.2 (BD Biosciences) and FlowJo vX (Tree Star Inc., Ashland, OR) software, respectively. We performed routine daily quality control tests with Cytometer Setup & Tracking Beads (BD Biosciences) in accordance with the manufacturer' s instructions. The optimal voltage ranges and linearity for photomultipliers were selected using 6-peak Rainbow Calibration Particles and Unstained Comp-Beads (BD Biosciences). All laboratory measurements were performed by staff blind to the clinical characteristics.

Statistical analysis

Categorical variables are expressed as percentages, and continuous variables are expressed as means ± standard deviation (SD) or medians (25th–75th percentiles) for normal and non-normal distributions, respectively. Data distributions were assessed with normal Q–Q plots.

Differences between groups were assessed with chi-squared test, Student's t-test and Mann-Whitney U test as appropriate, both for monocyte subset percentage and concentration. Correlations were assessed with Pearson test or Spearman Rho test as appropriate. Univariable Cox regression analyses were performed with all-cause death, HF-related hospitalization, and the composite end-point of both as the dependent variables and age, sex, ischemic etiology, New York Heart Association (NYHA) functional class, left ventricular ejection fraction (LVEF), hemoglobin, sodium, estimated glomerular filtration rate, NTproBNP (Log-transformed and per 1 SD), and monocyte subset percentages and absolute cell count (Log-

transformed and per 1 SD), as independent variables. Multivariable analyses were performed with the same dependent variables and the subsets of monocytes found to be statistically significant in the univariable analysis as independent variables, together with variables with $p<0.1$ in the univariable analysis and sex (considered clinically relevant) as co-variables. Colinearity between monocyte subsets was discarded by variance inflation factor (VIF). We adopted the Gray method of including competing risk for the analyses of HF-related hospital admission, considering death as the competing event for HF-related hospitalizations. Survival curves for all-cause death and the composite end-point, were plotted based on quartiles of intermediate monocyte subset absolute cell count. Statistical analyses were performed with SPSS 15 (SPSS Inc., Chicago, IL) and STATA V.13.0 (StataCorp LLC, College Station, TX,), and a two-sided $p<0.05$ was considered significant.

Results

400 patients were consecutively included in the study. The demographic and clinical characteristics of the patients at the data of sample collection relative to all-cause death are listed in Table 1. Briefly, enrolled patients were middle aged, predominantly males with ischemic systolic heart failure, typically NYHA functional class II or III (2 patients were NYHA class IV), with long-term HF duration, moderately depressed LVEF, and treated according to guideline-derived recommendations. During a mean follow-up of 2.6 ± 0.9 years, 107 patients died, 99 had a HF-related hospitalization and 160 suffered the composite end-point of all-cause death or HF-related hospitalization.

Within this cohort of HF patients, we comparatively assessed three monocyte subsets—referred to as classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{++}/CD16^{+}$), and nonclassical ($CD14^{+}/CD16^{++}$) (**Fig 1**). We did not find any correlation between subset percentage nor subset number of cells/μL and NYHA functional class, LVEF or estimated glomerular filtration rate (**S1 Table**), except for percentage of intermediate monocyte subset which showed a weak inverse correlation with LVEF (Rho –0.14, p = 0.004). When the monocyte subset distribution was assessed according to the different etiologies of HF, globally there were no statistically significant differences when percentages of subsets were considered, but a significant difference was observed in the non-classic subset when number of cells was measured (p = 0.04). In a specific comparison of such monocyte subset, valvular patients showed lower percentage (6.7 ± 3.4 *versus* 8.2 ± 4, p = 0.04) and number of cells [35.2 (23.7–63) *versus* 49.1 (34.7–70), p = 0.01] than ischemic patients and also than patients with dilated cardiomyopathy [8.3 ± 3.3, p = 0.03 and 49.7 (36.5–77.3), p = 0.006, respectively]. No differences were observed between ischemic patients and patients with dilated cardiomyopathy in any subset. No differences were also observed among etiologies in the intermediate monocyte subset.

Mean percentage and number of cells/μL of the monocyte subsets for the total cohort and for alive and deceased patients at the end of the study are summarized in Table 2. Significant differences were found between alive and deceased patients at the end of the study for the non-classical monocyte ($CD14^{+}/CD16^{++}$) subset considered as percentage, and in intermediate subset when absolute cell count (number of cells/μL) was considered (**Table 2**).

Furthermore, Table 3 shows the univariablpoorere Cox regression analyses for risk of all-cause death, HF-related hospitalization, and the composite end-point based on percentage and absolute cell count of monocyte subsets. S2 Table shows univariable analyses for several clinical variables. When considering percentages of monocyte subsets, non-classical monocyte subset showed protective significant association with all-cause death and the composite end-point in the univariable analysis. However, in the multivariable analyses, it did not remain related any of these end-points (**S3 Table**).

Table 1. Baseline demographic, clinical and biochemical data of the study participants.

	Total	Alive	Deceased	p-value
	n = 400	n = 293	n = 107	
Age (years)	69.4 ± 12.2	66.7 ± 11.9	76.9 ± 9.7	<0.001
Male sex	291 (72.8%)	213 (72.7%)	78 (72.9%)	0.97
Aetiology				0.006
Ischemic heart disease	182 (45.5%)	122 (41.6%)	60 (56.1%)	
Dilated CM	73 (18.3%)	62 (21.2%)	11 (10.3%)	
Hypertensive CM	35 (8.8%)	23 (7.8%)	12 (11.2%)	
Alcoholic CM	20 (5.0%)	18 (6.1%)	2 (1.9%)	
Drug-induced CM	14 (3.5%)	12 (4.1%)	2 (1.9%)	
Valvular disease	36 (9.9%)	25 (8.5%)	11 (10.3%)	
Hypertrophic CM	10 (2.5%)	10 (2.4%)	0 (0.0%)	
Other	30 (7.5%)	21 (7.2%)	9 (8.4%)	
HF duration (months)	72 (26–131)	69 (24–121)	81 (28–144)	0.08
Mean LVEF	41.6% ± 14.5	43.1% ± 13.9	37.5% ± 15.4	0.001
LVEF category				0.002
<40%	181 (45.3%)	118 (40.3%)	63 (58.9%)	
40–49%	103 (25.7%)	81 (27.6%)	22 (20.6%)	
≥50%	116 (29%)	94 (32.1%)	22 (20.6%)	
NYHA functional class				<0.001
I	24 (6.0%)	23 (7.8%)	1 (0.9%)	
II	251 (62.8%)	211 (72.0%)	40 (37.4%)	
III-IV	125 (31.2%)	59 (20.2%)	66 (61.7%)	
Co-morbidities				
Hypertension	294 (73.5%)	206 (70.3%)	88 (82.2%)	0.02
Diabetes mellitus	173 (43.3%)	122 (41.6%)	51 (47.7%)	0.28
COPD	89 (22.3%)	52 (17.7%)	37 (34.6%)	<0.001
Renal failure*	218 (54.5%)	126 (43.0%)	92 (86.0%)	<0.001
Anaemia#	167 (41.8%)	98 (33.4%)	69 (64.5%)	<0.001
Atrial fibrillation/flutter	170 (42.5%)	110 (37.5%)	60 (56.1%)	0.001
Biochemical				
Na	139 ± 3.6	139.5 ± 3.2	138.1 ± 4.2	<0.001
Haemoglobin	12.9 ± 1.7	13.2 ± 1.6	12.2 ± 1.7	<0.001
eGFR	58.3 ± 26.6	65.0± 26.4	40.2 ± 17.3	<0.001
NTproBNP	983 (311–2678)	672 (189–1573)	3436 (1617–6932)	<0.001
Treatments				
ACEI/ARB	331 (82.8%)	255 (87.0%)	76 (71.0%)	<0.001
Beta-blockers	359 (89.8%)	268 (91.5%)	91 (85.03%)	0.06
MRA	205 (51.3)	147 (50.2)	58 (54.2)	0.48
Loop diuretics	330 (82.5%)	224 (76.5%)	106 (99.1%)	<0.001
Digoxin	97 (24.3%)	57 (19.5%)	40 (37.46%)	<0.001
Ivabradine	37 (9.3%)	28 (9.6%)	9 (8.43%)	0.73
Statins	297 (74.3%)	217 (74.1%)	80 (74.8%)	0.89
ICD	85 (21.3%)	69 (23.5%)	16 (15.0%)	0.06

(*Continued*)

Table 1. (Continued)

	Total	Alive	Deceased	p-value
	n = 400	n = 293	n = 107	
CRT	63 (15.8%)	45 (15.4%)	18 (16.8%)	0.72

Data expressed as mean ± SD, median (25th–75th percentiles) or absolute number (percentage).

*eGFR (CKD-EPI) <60 mL/min/1.73m^2.

#Hb <12 g/dL in women and < 13g/dL in men.

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CM: cardiomyopathy; COPD, chronic obstructive pulmonary disease; CRT, cardiac resynchronization therapy; eGFR, estimated glomerular filtration rate; ICD, implantable cardioverter device; LVEF, left ventricular ejection fraction; MRA: mineral corticoid receptor antagonist; NTproBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association.

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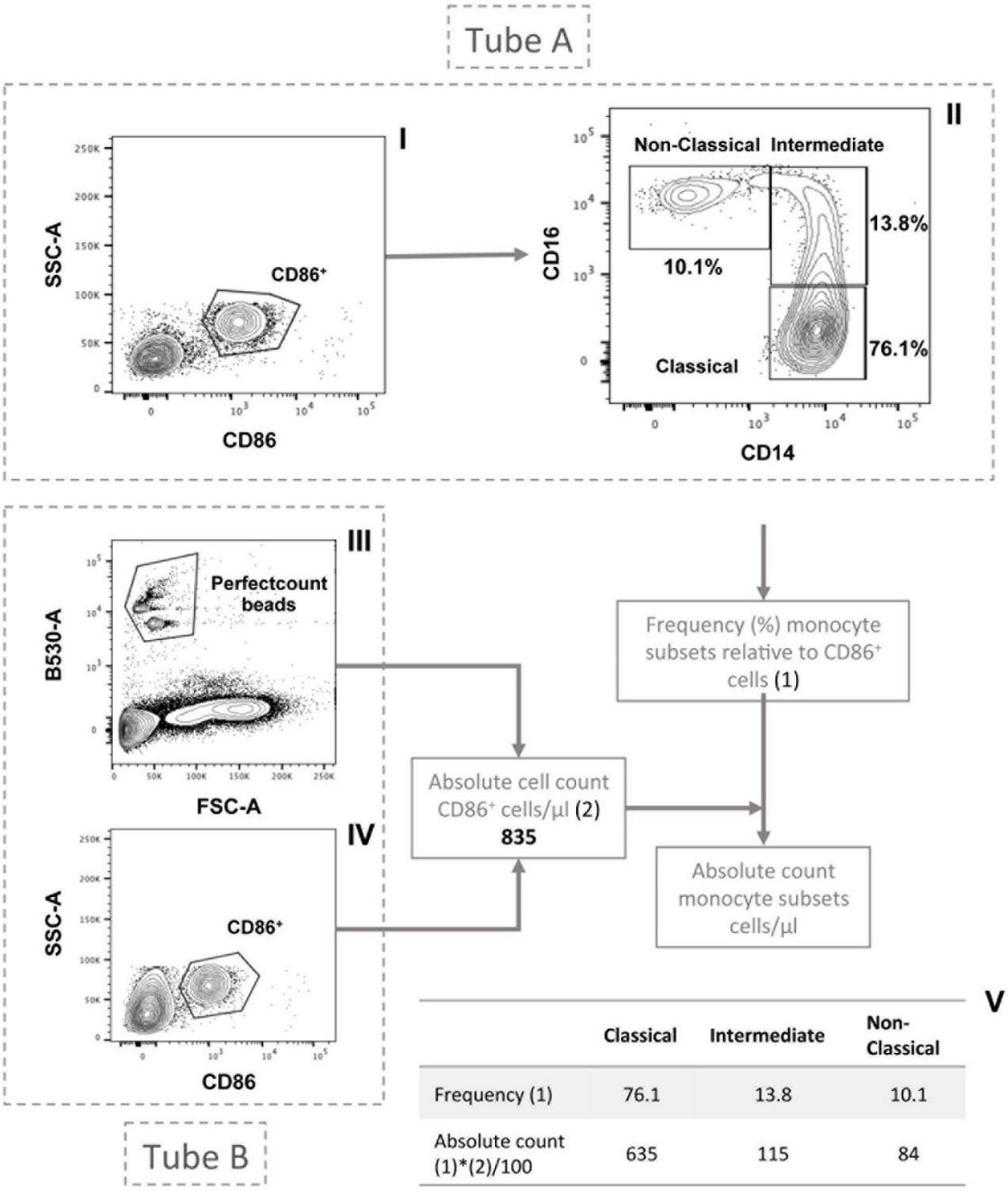
By contrast, when considering absolute cell count, the intermediate ($CD14^{++}/CD16^{+}$) monocyte subset showed detrimental association with all-cause death and the composite end-point and showed borderline significance with HF-related hospitalization, while the non-classical ($CD14^{+}/CD16^{++}$) subset showed protective association with all-cause death and borderline significance with the composite end-point in the univariable analyses (**Table 3**). Fig 2 depicts free-event survival curves for quartiles (number of cells/μL) for $CD14^{++}/CD16^{+}$ monocytes. The fourth quartiles showed a 58% increase in the risk of suffering a composite end-point relative to the first quartile (p = 0.04), and Fig 3 depicts survival curves for the same quartiles. Fourth quartile showed an 87% increase in the risk of all-cause death relative to first quartile (p = 0.02). In multivariable analyses, however, only the intermediate monocyte subset remained associated with all-cause death (p = 0.03) and with the composite end-point (p = 0.02) (**Table 4**).

Discussion

Monocytes can have both damage and repair functions in different cardiovascular diseases, depending on the subset. In healthy individuals, monocyte subset distribution and function showed a significant prevalence of classical ($CD14^{++}/CD16^{-}$) monocyte subset (80.1±7%) in comparison to the other two subsets defined as intermediate ($CD14^{++}/CD16^{+}$; 3.7±2.0%) and non-classical ($CD14^{+}/CD16^{++}$; 6.2±2.8%) [20]. Different distributions of monocyte subsets in disease contexts may be related to pathogenesis and the specific functions of classical, intermediate, and non-classical monocytes [21].

We investigated two main questions in this study: first, what is the distribution of the three subsets of monocytes in outpatients with HF diagnosis, and second, can this distribution be related to the main events of the study, and therefore play predictive role for HF patients? In this context, we found a significant difference in the percentages of monocyte subsets relative to previous studies performed in healthy controls [20]. The predominant monocyte subset was the classical (50.0±17.2%), followed by the intermediate (42±17.2%) subset; the non-classical subset of monocytes occurred with much less frequency (8.1±4.0%). This represents a significant expansion of the intermediate subset in HF in comparison with healthy controls.

The $CD14^{+}/CD16^{+}$ intermediate subset is not yet completely understood. It has been reported to have characteristics similar to both the classical and non-classical monocytes (high phagocytic activity, antigen presentation and T-cell interaction [22–24]), and some studies have suggested that it may represent a stage of differentiation to immature classical and non-classical subsets [23]. The intermediate subset has been associated with many clinical conditions, ranging from chronic inflammation and type 2 diabetes mellitus to chronic vascular and endothelial damage and atherosclerosis [14, 25, 26]. Intermediate monocytes have a more pro-



V

	Classical	Intermediate	Non-Classical
Frequency (1)	76.1	13.8	10.1
Absolute count (1)*(2)/100	635	115	84

Fig 1. Assessment of peripheral blood monocyte subsets by flow cytometry. In tube A, total monocyte population and frequency of the monocyte subsets was defined with CD86 (**I**) and CD14/CD16 (**II**) expression, respectively. In tube B, Perfect count beads (**III**) and monocytes ($CD86^+$ cells) (**IV**) were selected to determine total monocyte cell count. Frequency was then applied to total monocyte count in order to measure monocyte subsets (**V**). Table summarizes representative results from one patient with ambulatory heart failure.

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Table 2. Percentage and concentration of circulating monocyte subsets.

	Total	Alive	Deceased	p-value
Percentage	**N = 400**	**N = 293**	**N = 107**	
$CD14^{++}/CD16^{-}$	50.0 ± 17.2	50.4 ± 16.5	48.9 ± 19.08	0.45
$CD14^{++}/CD16^{+}$	42.0 ± 17.2	41.2 ± 16.5	44.0 ± 18.8	0.15
$CD14^{+}/CD16^{++}$	8.1 ± 4.0	8.42 ± 4.0	7.1 ± 4.0	0.005
Number of cells/μL				
$CD14^{++}/CD16^{-}$	330 (223–441)	327 (222–435)	363 (227–451)	0.38
$CD14^{++}/CD16^{+}$	258 (172–393)	253 (170–374)	303 (186–470)	0.02
$CD14^{+}/CD16^{++}$	47 (34–71)	48 (35–71)	44 (27–73)	0.10

Data expressed as mean ± SD or median (Q1-Q3).

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inflammatory capacity than the non-classical subset [27], as they secrete more oxygen radicals, TNF-α, and IL-1β [26]. Collectively, this may explain the elevated levels of intermediate monocytes in our HF cohort, in which the majority of the patients were men with chronic HF with ischemic aetiology. For instance, the intermediate subset experienced a significant dynamic elevation of its blood levels in ST-elevation myocardial infarction, which correlated with troponin elevation and left ventricular function [24]. To our knowledge, there is only one study reporting the distribution of monocytes in patients with acute and stable ischemic HF [10], but conclusive evidence of the significance and importance of the monocyte distribution in patients with chronic HF is still not yet well understood. The intermediate subset was increased in patients with both acute and stable HF in the previous studies [28], as seen in our cohort in comparison to healthy individuals. In this context, the maintenance of cardiac integrity through removal of irreparably dead cells is crucial. An influx of pro-inflammatory cells such as intermediate monocytes into the damaged area is thought to be essential for the very early wound healing process, but their persistence beyond the initial repair phase could extend longer-term inflammation-related adverse effects into healthy remote myocardial areas. This could be a potential mechanistic explanation of the poorer prognosis of HF patients with higher intermediate monocytes.

A significant difference was found for the intermediate subsets when number of cells/μL was addressed. Remarkably, although patients who died had worse clinical characteristics

Table 3. Univariable Cox regression analysis for risk of all-cause death, HF-related hospitalization, and the composite end-point all-cause death or HF hospitalization, based on percentage and cells/μL of monocyte subsets.

	All-cause death			HF-related hospitalization*			Composite end-point		
	HR	**[95% CI]**	**p-value**	**HR**	**[95% CI]**	**p-value**	**HR**	**[95% CI]**	**p-value**
Percentage									
$CD14^{++}/CD16^{-}$	1.00	[0.98–1.01]	0.38	1.00	[0.98–1.01]	0.52	1.00	[0.99–1.01]	0.53
$CD14^{++}/CD16^{+}$	1.01	[1.00–1.02]	0.12	1.00	[0.99–1.01]	0.44	1.01	[1.00–1.02]	0.23
$CD14^{+}/CD16^{++}$	0.93	[0.88–0.98]	0.004	0.99	[0.94–1.04]	0.66	0.95	[0.91–0.99]	0.02
Number of cells/μL#									
$CD14^{++}/CD16^{-}$	1.07	[0.88–1.31]	0.49	1.04	[0.84–1.29]	0.71	1.05	[0.90–1.24]	0.54
$CD14^{++}/CD16^{+}$	1.29	[1.06–1.56]	0.01	1.18	[0.99–1.41]	0.07	1.18	[1.01–1.38]	0.04
$CD14^{+}/CD16^{++}$	0.83	[0.70–0.99]	0.04	1.00	[0.81–1.23]	0.99	0.87	[0.75–1.01]	0.08

*Death has been considered as competitive risk for HF-related hospitalization.

#Log-transformed and per 1 SD.

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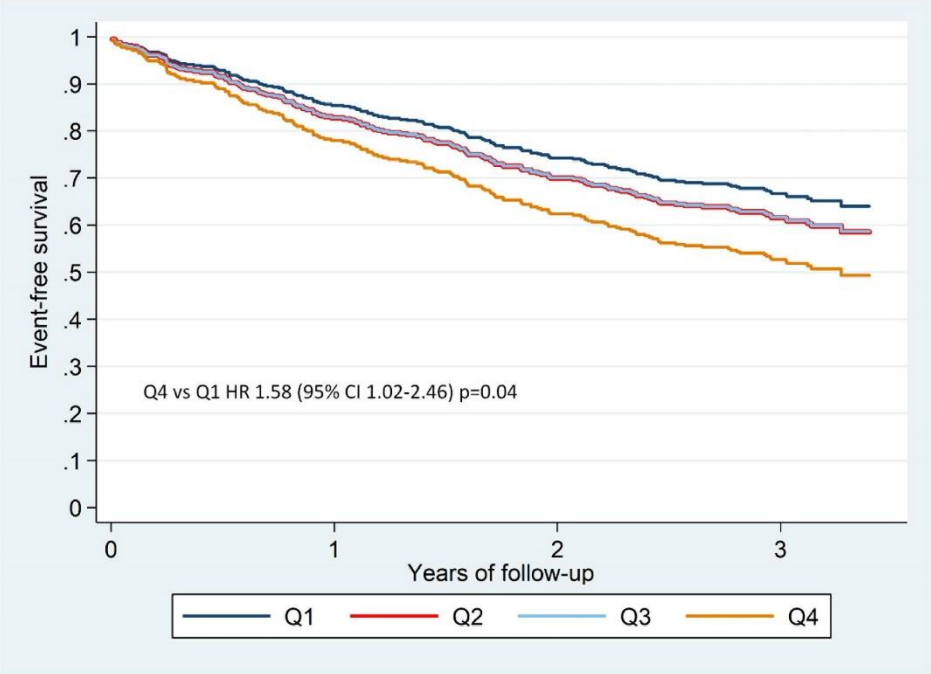

Fig 2. Event-free survival curves for the composite end-point of all-cause death or HF-related hospitalization, relative to quartiles of number of cells/μL of the intermediate ($CD14^{++}/CD16^{+}$) monocyte subset. HR Q4 *versus* Q1: 1.58 (95% CI 1.02–2.46), p = 0.04).

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(higher age, worse NYHA functional class, worse LVEF, higher NTproBNP, worse renal function) the intermediate subset was independently associated with all-cause death and the composite end-point in the multivariable analyses. Wrigley *et al.* previously reported that the expanded intermediate subset was associated with poorer prognosis in patients with acute HF [10]. It should be emphasized that our cohort is an ambulatory chronic cohort, with much less inflammation activation than seen in acute HF, but we also found such prognostic relationship. Other studies have indicated that the intermediate subset proved to be risk factor for post-ST elevation myocardial infarction adverse outcomes and cardiovascular events [29, 30]. Also, the amount of intermediate monocytes was found to correlate with worse cardiac function and predicted the possibility to reach an improvement in NYHA functional class at 3 months after transcatheter aortic valve replacement [31].

An additional relevant finding in this study is the assessment and prognostic value of monocyte subset absolute cell count, beyond the classical approach of subset percentages. Monocyte cell count (defined as number of cells/μL) has been assessed in previous studies of healthy patients [20]. We found that the absolute count was markedly superior to the percentage distribution of each subset when considering the prognostic role of the different monocyte subsets. An increase or decrease in subpopulation percentage may reflect a change in another cell subtype in addition to the subset of interest, and this issue could be resolved by using absolute values combined with relative values. Absolute cell counts have been shown to be a major tool for laboratory diagnosis for a variety of pathological conditions, including $CD4^{+}$ T-cells for Acquired ImmunoDeficiency Syndrome and haematopoietic progenitor cells expressing

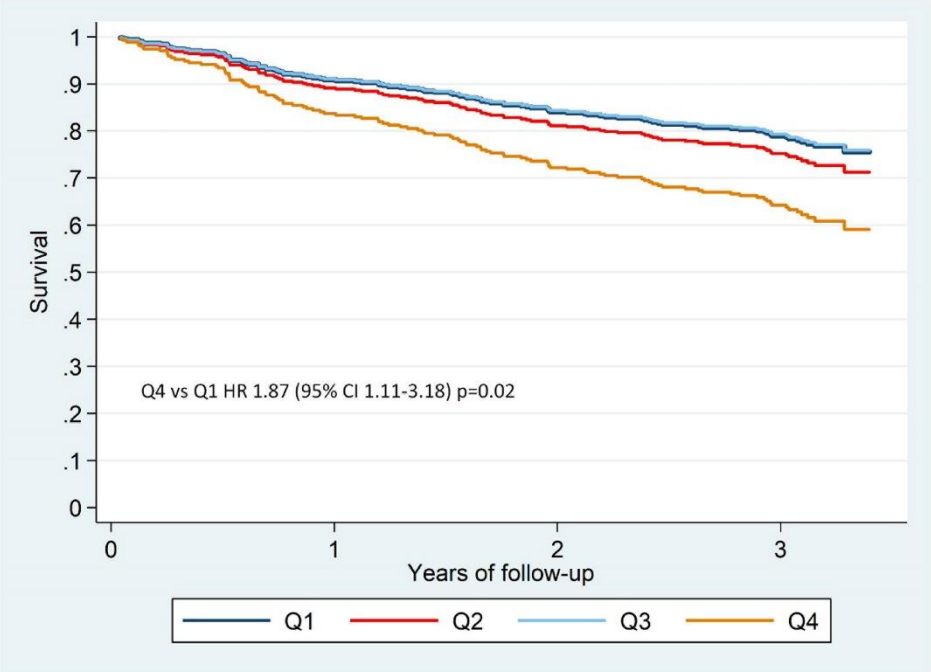

Fig 3. Survival curves for all-cause death, relative to quartiles of number of cells/μL of the intermediate ($CD14^{++}/CD16^{+}$) monocyte subset. HR Q4 *versus* Q1: 1.87 (95% CI 1.11–3.18), p = 0.02.

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CD34 for cord blood transplants and autotransplantation [32, 33]. T-cell count imbalances have also been described in association with protein-caloric malnutrition, anorexia nervosa, lactation and iron deficiency, autoimmune thyroiditis, bronchial asthma, multiple sclerosis, polymyalgia rheumatica, and rheumatoid arthritis [34].

Table 4. Multivariable Cox regression analysis for risk of all-cause death, HF-related hospitalization, and the composite end-point all-cause death or HF hospitalization, including number of cells/μL of $CD14^{++}CD16^{+}$ (intermediate) and $CD14^{+}/CD16^{++}$ (non-classic) monocyte subsets when appropriate.

	All-cause death			HF-related hospitalization*			Composite end-point		
	HR	[95% CI]	p-value	HR	[95% CI]	p-value	HR	[95% CI]	p-value
$CD14^{++}/CD16^{+}$, #	1.25	[1.02–1.52]	0.03	—	—	—	1.20	[1.03–1.40]	0.02
$CD14^{+}/CD16^{++}$, #	—	—	—				—	—	—
Age	1.04	[1.02–1.06]	<0.001	—	—	—	1.02	[1.00–1.03]	<0.05
Female sex	—	—	—	—	—	—	—	—	—
NYHA functional class	2.17	[1.45–3.25]	<0.001	—	—	—	2.21	[1.60–3.06]	<0.001
LVEF	—	—	—	—	—	—	—	—	—
Haemoglobin	—	—	—	—	—	—	—	—	—
Sodium	0.94	[0.89–0.99]	0.01	—	—	—	—	—	—
eGFR	—	—	—	—	—	—	—	—	—
NTproBNP#	2.69	[2.06–3.51]	<0.001	2.10	[1.68–2.61]	<0.001	2.17	[1.78–2.66]	<0.001

* Death has been considered as competitive risk for HF-related hospitalization

Log-Transformed and per 1 SD. eGFR, estimated glomerular filtration rate

HF, heart failure; LVEF, left ventricular ejection fraction; NTproBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association.

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The possibility of selection bias represents a potential limitation of the study. The participants reported here were drawn from a general population that visited our tertiary hospital HF Clinic. The majority had been admitted to the hospital in previous years and the cohort included primarily male patients with ischemic heart disease as the main cause of HF. We have analyzed only one blood sample per patient, and cannot comment on the prognostic value of serial determinations. Indeed, although blood samples were obtained in routine ambulatory visits, we cannot discard that in some isolated patient it could have been obtained after a relatively near inciting incident (i.e. exacerbation) which could have mobilized the cells of interest. We did not have data on other inflammatory cytokines such as TNF, IL6, or IL1b, which might could yield insights into the mechanistic role of differential monocyte distribution.

Conclusion

This investigation evaluated the relationship between circulating monocyte subsets and HF. The classical ($CD14^{++}/CD16^{-}$) monocyte subset was decreased and the intermediate ($CD14^{++}/CD16^{+}$) monocyte subset was increased in patients with HF compared to reported controls. The quantification of the absolute cell count of each monocyte subset (number of cells/μL) showed a superior prognostic value for the studied cohort of patients, compared to the performance of monocyte subset analysis by percentages. Moreover, the intermediate subset was independently associated with all-cause death and the composite end-point of all-cause death or HF hospitalization, in multivariable analyses. Indeed, further studies are necessary to confirm our preliminary findings and to better underscore the clinical value of these data.

Supporting information

S1 Table. Correlations among monocyte subsets and clinical variables.
(DOCX)

S2 Table. Univariable Cox regression analysis for risk of all-cause death, HF-related hospitalization, and the composite end-point all-cause death or HF-related hospitalization based on clinical variables.
(DOCX)

S3 Table. Multivariable Cox regression analysis for risk of all-cause death, HF-related hospitalization, and the composite end-point all-cause death or HF hospitalization, including percentage of the $CD14^{+}/CD16^{++}$ (non-classic) monocyte subset.
(DOCX)

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Funding acquisition: Antoni Bayés-Genís.

Methodology: Iris Teubel.

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Writing – review & editing: Carolina Gálvez-Montón, Marta de Antonio, Pedro Moliner, Mar Domingo, Elisabet Zamora, Julio Núñez, Germán Cediel.

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5.3. Third study

Title:

Telomere attrition in heart failure: a flow-FISH longitudinal analysis of circulating monocytes.

Authors:

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RESEARCH Open Access

Telomere attrition in heart failure: a flow-FISH longitudinal analysis of circulating monocytes

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Abstract

Background: Cross-sectional investigations report shorter telomeres in patients with heart failure (HF); however, no studies describe telomere length (TL) trajectory and its relationship with HF progression. Here we aimed to investigate telomere shortening over time and its relationship to outcomes.

Methods: Our study cohort included 101 ambulatory patients with HF. Blood samples were collected at baseline (n = 101) and at the 1-year follow-up (n = 54). Using flow-FISH analysis of circulating monocytes, we simultaneously measured three monocyte subsets—classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and nonclassical ($CD14^{+}CD16^{++}$)—and their respective TLs based on FITC-labeled PNA probe hybridization. The primary endpoints were all-cause death and the composite of all-cause death or HF-related hospitalization, assessed at 2.3 ± 0.6 years. All statistical analyses were executed by using the SPSS 15.0 software, and included Student's t test and ANOVA with post hoc Scheffe analysis, Pearson or Spearman rho correlation and univariate Cox regression when applicable.

Results: We found high correlations between TL values of different monocyte subsets: $CD14^{++}CD16^{+}$ vs. $CD14^{++}CD16^{-}$, $R = 0.95$, $p < 0.001$; $CD14^{++}CD16^{+}$ vs. $CD14^{+}CD16^{++}$, $R = 0.90$, $p < 0.001$; and $CD14^{++}CD16^{-}$ vs. $CD14^{+}CD16^{++}$, $R = 0.89$, $p < 0.001$. Mean monocyte TL exhibited significant attrition from baseline to the 1-year follow-up (11.1 ± 3.3 vs. 8.3 ± 2.1, $p < 0.001$). TL did not significantly differ between monocyte subsets at either sampling time-point (all p values > 0.1). Cox regression analyses did not indicate that TL or ΔTL was associated with all-cause death or the composite endpoint.

Conclusions: Overall, this longitudinal study demonstrated a ~ 22% reduction of TL in monocytes from ambulatory patients with HF within 1 year. TL and ΔTL were not related to outcomes over long-term follow-up.

Keywords: Heart failure, Monocyte subsets, Telomere attrition, Telomere length

Background

Heart failure (HF) has become an epidemic, imposing substantial health, social, and economic burdens. In developed countries, HF affects 1–2% of the adult population, with a prevalence of ≥ 10% among those ≥ 70 years of age [1]. It has been suggested that HF is a disease of accelerated aging, and telomere length (TL) is proposed as a biomarker of aging [2]. Telomeres are specialized and evolutionarily conserved tandem repeats (5′-TTAGGG-3′ in humans) located at the end of chromosomes. They serve as protective caps that prevent the DNA damage-repair system from accidentally identifying chromosomal ends as DNA double strands [3, 4]. Preliminary data from cross-sectional studies reveal that persons with HF have shorter telomeres than healthy age- and gender-balanced controls, based on analyses of circulating leukocytes using a conventional quantitative polymerase chain reaction (qPCR)-based method [5].

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However, no longitudinal studies have investigated telomere attrition among patients with chronic HF.

TL can also be efficiently assessed in monocytes, a heterogeneous population of effector cells that play key roles in maintaining and restoring tissue integrity [6]. Using flow cytometry, circulating monocytes can be categorized into three distinct subsets based on differential expression levels of the surface markers CD14 and CD16: classic, $CD14^{++}CD16^{-}$; intermediate, $CD14^{++}CD16^{+}$; and non-classic, $CD14^{+}CD16^{++}$ [7]. We recently reported TL assessment in monocyte subsets using a novel standardized analytical protocol based on simultaneous multicolor flow cytometry-fluorescence in situ hybridization (flow-FISH) [8].

In the present article, we report a longitudinal study in which we used the novel flow-FISH technique to explore the dynamics of telomere attrition in circulating monocytes within a cohort of ambulatory HF patients.

Methods

Study population

Our study cohort included 101 ambulatory patients who attended a multidisciplinary HF unit from January 15th 2014 to May 6th 2015 (Table 1). The referral inclusion criteria are described elsewhere [9, 10]. All patients made follow-up visits at regular predefined intervals, and additional visits when required in cases of decompensation. The regular visitation schedule included a minimum of quarterly visits with nurses; biannual visits with physicians; and elective visits with geriatricians, psychiatrists, nephrologists, and rehabilitation physicians. Upon missing a regular visit, patients were contacted by telephone.

The primary endpoints were all-cause death and the composite of all-cause death or HF-related hospitalization. Fatal events were identified from electronic clinical records, and by contacting the patients' relatives when necessary. When verification was required, data were compared with records stored in the databases of the Catalan and Spanish health systems. Events were adjudicated by two of the authors (EE and JL), and by clinical and research nurses.

Each subject gave their written informed consent prior to participation. The study protocol was approved by the Clinical Research Ethics Committee of our institution, was designed in accordance with the principles outlined in the 2013 revision of the Declaration of Helsinki of 1975 [11].

Blood extraction and processing

Blood samples of ~ 3 ml were collected into EDTA tubes via standard forearm venipuncture performed between 9:00 a.m. and 11:00 a.m., and were processed within 4 h after collection. Samples were collected at two time-points: at baseline (n = 101) and at the 1-year follow-up (n = 54) (Additional file 1: Table S1). Samples from the 1-year follow-up were unavailable due to death (7 patients), technical issues (10 patients), or patient's unwillingness to repeat sampling (30 patients). Samples and data from patients included in this study were processed and collected by the IGTP-HUGTP Biobank integrated in the Spanish National Biobanks Network of Instituto de Salud Carlos III (PT13/0010/0009) and Tumour Bank Network of Catalonia. All laboratory measurements were performed by staff blinded to the patients' clinical characteristics.

Flow–FISH

Blood samples were first lysed by a 10-min incubation with PharmLyse solution (BD Bioscience, San Diego, CA, USA), and then the cell concentration was measured by flow cytometry using Perfect-Count beads (Cytognos, Salamanca, Spain). In a 15-min incubation at room temperature (RT), 1×10^6 cells were stained with titrated amounts of the following antibodies: CD86-BV605, CD14-BV785 (Biolegend, San Diego, CA, USA), CD16-BV421, and CD15-AlexaFluor647 (BD Biosciences). Next, these cells were fixed with 6 mM bis(sulfosuccinimidyl) suberate (Sigma-Aldrich Química SL, Madrid, Spain) for 30 min at 2–8 °C. The reaction was quenched using 1 M Tris buffer (pH 8.0) for 15 min at RT. Then the residual red blood cells were removed by incubation with FACS lysing solution (BD Biosciences) for 7 min at RT.

FISH was performed using the Telomere PNA kit (Dako, Glostrup, Denmark) following the manufacturer's instructions. The human 4-year old Caucasian female acute lymphoblastic leukemia 1301 cell line from the Health Protection Agency Culture Collections (HPACC) was used with each sample as an internal control. The 1301 cell line was previously cultured according to HPACC recommendations in RPMI 1640 (Gibco, Life Technologies, Grand Island, NY) with 10% fetal bovine serum (Sigma-Aldrich Química SL), penicillin, streptomycin, and glutamine (Gibco, Life Technologies). After purchase of the 1301 cell line, subsequent cells were obtained from four passages after reaching a maximum of 1×10^6 cells viable cells/ml in culture; cells with the same passage number were aliquoted in large numbers and stored at − 196 °C until use. Samples were acquired by flow cytometry, with up to 10,000 monocytes collected per sample. We performed correction for DNA ploidy of the blood sample vs. the internal control as previously described [12].

All samples were acquired on a Fortessa SORP flow cytometer (BD Biosciences) equipped with four lasers (100-mW 488 nm, 150 mW 532 nm, 50 mW 405 nm, and 100 mW 640 nm) using sample acquisition software

FACSDiva v6.2 (BD Biosciences) and analyzed with FlowJo vX (Tree Star, Inc, Ashland, OR). We performed routine daily quality control tests with Cytometer Setup & Tracking Beads (BD Biosciences) in accordance with the manufacturer's instructions. Daily QC control of 6-peak Rainbow Calibration Particles (BD Biosciences) was used for Flow-FISH MFI standardization to reach initial target MFI values. We initially gated for G0/G1 cells of both leukocyte subsets and 1301 cells based on DNA content, and then by scatter properties. Monocytes were sequentially identified using a CD86 vs. CD16 plot, followed by a CD15 vs. CD16 plot to gate out neutrophils. Next, the gated monocytes were analyzed for CD14 and CD16 expression. Clumped cells were excluded using a plot of propidium iodide (PI) area vs. PI width. Finally, each subset of monocytes and internal control cells was displayed on a plot comprising the FITC-labeled PNA probe on PI (B695-A) vs. the PNA probe (B515-A), and the median fluorescence intensity (MFI) of the PNA probe was measured.

The relative TL value for each monocyte subset was calculated as the ratio between the MFI of each subset and the MFI of the control cells. Corrections were made for the DNA index of G0/G1 cells, as previously described [13].

Statistical analysis

Categorical variables are expressed as percentages. For continuous variables, data distributions were assessed using normal Q–Q plots, and data are expressed as mean and (SD) for normally distributed data, or as median and (quartiles Q1–Q3) for non-normally distributed data. Between-group differences were assessed using Student's t test and ANOVA with post hoc Scheffe analysis. To assess correlations among the TL of the different monocyte subsets, and between the mean TL of all monocytes and clinical variables, we used the Pearson or Spearman rho correlation test, as appropriate. Comparison between mean TL between baseline and 1-year samples were performed with t test for paired data.

We additionally performed univariate Cox regression analyses with all-cause death and the composite endpoint as the dependent variables, and with the mean TL for monocytes as a whole and for each monocyte subset as the independent variables. In the subgroup of patients for whom a 1-year follow-up blood sample was available, we also assessed the relative TL change using the formula [TL at 1 year –baseline TL]/baseline TL) × 100. Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). A two-sided p value of < 0.05 was considered significant.

Results

Table 1 shows the clinical characteristics of the studied population. In general, the patients were middle-aged and predominantly male, showed an ischemic etiology, and were NYHA functional class II or III and treated following contemporary guidelines.

Table 1 Baseline characteristics of the study participants

	n = 101
Age (years)	65.6 ± 11.3
Male sex	76 (75.2%)
Etiology	
Ischemic heart disease	46 (45.5%)
Dilated cardiomyopathy	18 (17.8%)
Hypertensive cardiomyopathy	8 (7.9%)
Alcoholic cardiomyopathy	7 (6.9%)
Valvular disease	8 (7.9%)
Hypertrophic cardiomyopathy	4 (4.0%)
Other	10 (9.9%)
HF duration in months	38.7 (12.7–77.4)
LVEF	41.8% ± 12.1
NYHA functional class	
I	9 (8.9%)
II	70 (69.3%)
III	22 (21.8%)
Co-morbidities	
Hypertension	74 (73.3%)
Diabetes mellitus	50 (49.5%)
Renal failure[a]	48 (47.5%)
Anemia[b]	41 (40.6%)
Atrial fibrillation/flutter	42 (41.6%)
Obesity	31 (30.7%)
Smoker	
Current	4 (4.0%)
Past	66 (65.3%)
Treatments	
ACEI/ARB	89 (88.1%)
Beta-blockers	93 (92.1%)
MRA	63 (62.4%)
Loop diuretics	81 (80.2%)
Digoxin	21 (20.8%)
Ivabradine	19 (18.8%)
Statins	86 (85.1%)
ICD	3 (3.0%)
CRT	16 (15.8%)

Data expressed as mean ± standard deviation, median (25th–75th percentiles), or absolute number (percentage)

ACEI angiotensin-converting enzyme inhibitor, *ARB* angiotensin receptor blocker, *CRT* cardiac resynchronization therapy, *ICD* implantable cardioverter device, *LVEF* left ventricular ejection fraction, *MRA* mineral corticoid receptor antagonist, *NYHA* New York Heart Association

[a] eGFR < 60 ml/min/1.73 m^2

[b] Hb of < 12 g/dl in women and < 13 g/dl in men

Within this cohort of HF patients, we measured three monocyte subsets—referred to as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and nonclassical ($CD14^{+}CD16^{++}$)—along with their respective TLs. The coefficient of variation of the intra-assay for duplicates was 5.08% for monocyte population with minor differences between subsets. Across all runs, inter-assay coefficient of variation was 71.82% for unprobed and 22.81% for probed, similar to previously described measurements by other groups [14]. Figure 1 shows a representative TL analysis of a whole-blood sample from a patient with HF. Collectively, we found that the TL values were highly correlated between the different monocyte subsets: $CD14^{++}CD16^{+}$ vs.

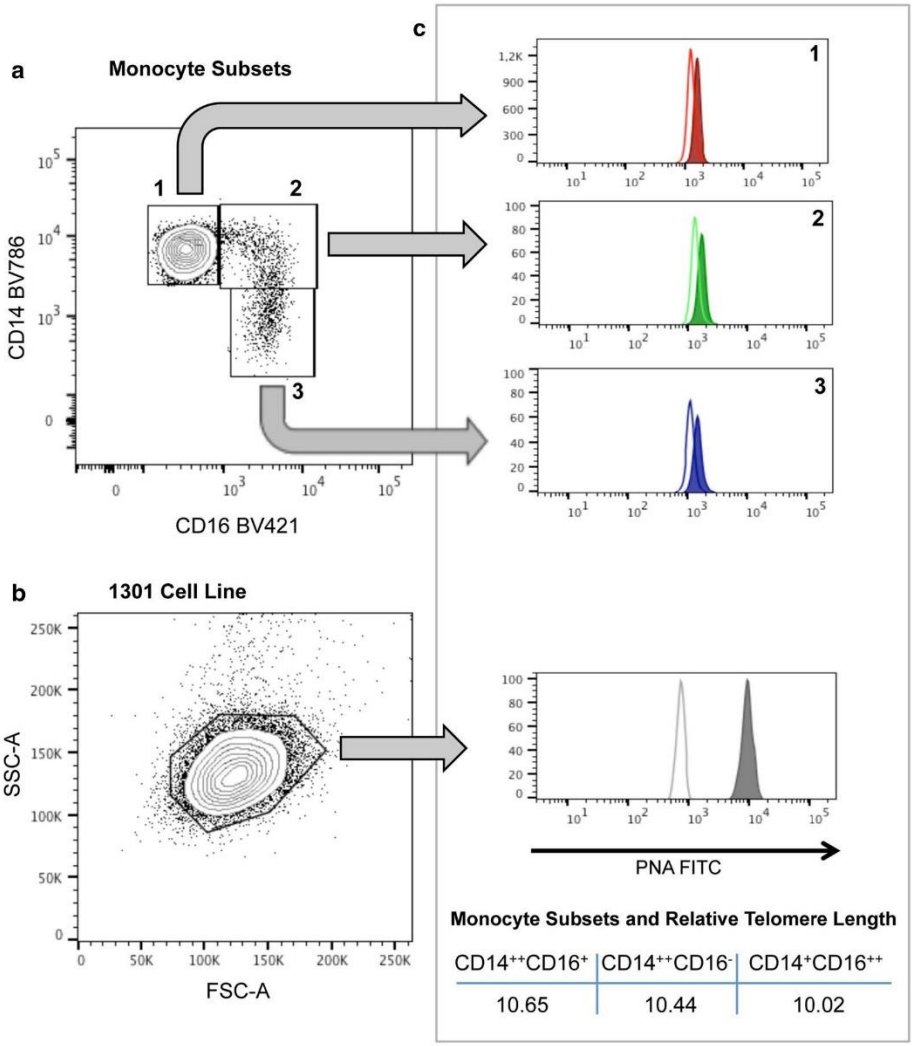

Fig. 1 Representative flow-FISH analysis of TL in monocyte subsets from an ambulatory patient with HF. Cells were previously selected by G0/G1 DNA Content, scatter properties and CD86 and CD15 markers for monocytes and 1301 cell line selection as described in Materials and methods (data not shown). Monocyte subsets were analyzed based on their CD14 and CD16 expression (**a**: 1. $CD14^{++}CD16^{-}$; 2. $CD14^{++}CD16^{+}$; 3. $CD14^{+}CD16^{+}$). Scatter properties of 1301 cell line and gating was shown in **b**. Hybridization of FITC-labeled Telomere Probe (PNA; color filled histograms) and control (without probe; empty histograms) in the three existing monocyte subsets and internal reference control 1301 cell line **c**. The relative TL value for each monocyte subset was finally calculated as the ratio between the MFI of each subset and the MFI of the control cells. Corrections were also made for the DNA index of G0/G1 cells

$CD14^{++}CD16^{-}$, R = 0.95, p < 0.001; $CD14^{++}CD16^{+}$ vs. $CD14^{+}CD16^{++}$, R = 0.90, p < 0.001; and $CD14^{++}CD16^{-}$ vs. $CD14^{+}CD16^{++}$, R = 0.89, p < 0.001. Table 2 shows TL at baseline and at the 1-year follow-up, as well as the percent change, for the whole-blood sample of monocytes and for each monocyte subset. Within the subgroup of patients for whom blood samples were available for both time-points, we found statistically significant ~ 22% attrition of mean monocyte TL (11.1 ± 3.3 vs. 8.3 ± 2.1, p < 0.001). Mean monocyte TL change was −22% ± 20 (Fig. 2 and Table 2). Monocyte TL reduction occurred in 96.3% of patients (ranging from − 3.1 to − 66.7%); in 28 of these patients (51.9%) monocyte TL reduction was ≥ − 20%. TL at baseline, TL at the 1-year follow-up, and the change in TL over 1 year did not significantly differ between the monocyte subsets (all p values > 0.1). In particular, there was no difference in TL attrition between genders (− 23.5 ± 14.4 and − 21.4 ± 22.5% in women and men, respectively). Furthermore no relationship between TL attrition and age (< 70 years vs. older), sex, ischemic etiology, presence of diabetes mellitus or HF duration (< 48 months vs. longer) was found.

Mean monocyte TL was not significantly related to any demographic or clinical characteristics, except for the presence of atrial fibrillation (p = 0.01) while a tendency to inverse correlation was observed with age (p = 0.09) (Table 3). Over the mean follow-up of 2.3 ± 0.6 years, 17 patients died, 17 required hospital admission due to HF, and 29 suffered the composite end-point of death or HF hospitalization. Table 4 shows Cox regression analyses for all-cause death and for the composite end-point of all-cause death or HF hospitalization. These outcomes were not associated with baseline TL or with change in TL over 1 year.

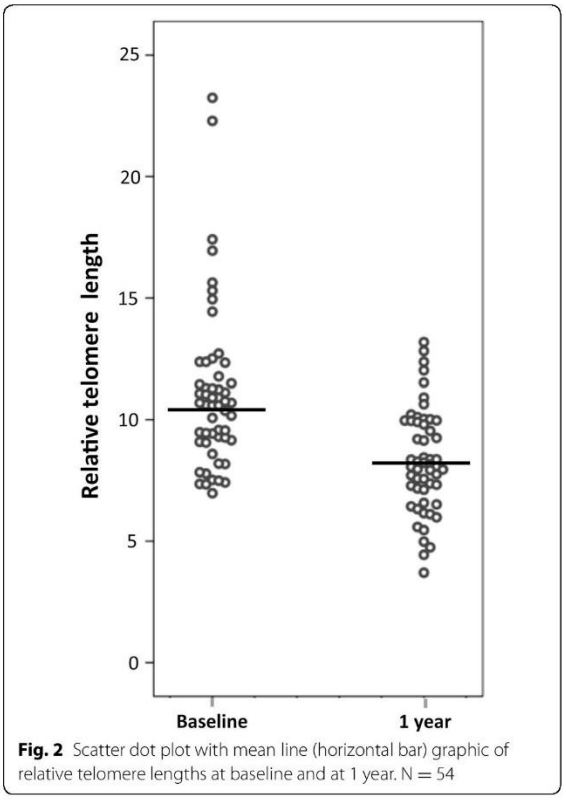

Fig. 2 Scatter dot plot with mean line (horizontal bar) graphic of relative telomere lengths at baseline and at 1 year. N = 54

Table 2 Telomere lengths at baseline and at 1 year

Baseline	**n = 101**
Monocytes, whole	10.3 ± 3.3
$CD14^{++}CD16^{-}$	10.5 ± 3.6
$CD14^{++}CD16^{+}$	10.1 ± 3.3
$CD14^{+}CD16^{++}$	10.3 ± 3.1
1 year	**N = 54**
Monocytes, whole	8.3 ± 2.1
$CD14^{++}CD16^{-}$	8.3 ± 2.2
$CD14^{++}CD16^{+}$	8.4 ± 2.3
$CD14^{+}CD16^{++}$	8.3 ± 2.1
%, Δ	**N = 54**
Monocytes, whole	− 22 ± 20
$CD14^{++}CD16^{-}$	− 25 ± 20
$CD14^{++}CD16^{+}$	− 19 ± 23
$CD14^{+}CD16^{++}$	− 21 ± 22

Data expressed as mean ± standard deviation

Discussion

The results of this longitudinal study revealed that TL significantly declined (by ~ 22%) within a year in circulating monocytes from patients with HF. Our data did not indicate that TL was correlated with various monocyte subsets or with HF outcomes. To our knowledge, this is the first study to describe the course of telomere length change within a well-characterized cohort of patients with HF using simultaneous flow-FISH.

Telomere biology is linked to aging and age-associated pathologies, and preliminary data from cross-sectional studies reveal shorter telomeres in HF patients compared to healthy age- and gender-balanced controls, based on measurements in circulating leukocytes using a conventional qPCR method [5]. Moreover, TL is reportedly associated with the severity of HF symptoms and outcome [5, 15], and with worse renal function in subjects with HF, which is a powerful predictor of outcome [16, 17]. While TL is usually measured in leukocytes, it has also been evaluated in cardiac tissue from patients with

Table 3 Baseline monocyte Telomere lengths according to demographic and clinical characteristics

	n = 101	p
Age (years)	R = − 0.17	0.09
Sex		0.44
Male	10.2 ± 2.9	
Female	10.7 ± 4.2	
Etiology		0.90[§]
Ischemic heart disease	10.4 ± 3.5	
Dilated cardiomyopathy	10.3 ± 4.4	
Hypertensive cardiomyopathy	10.5 ± 3.0	
Alcoholic cardiomyopathy	9.5 ± 1.8	
Valvular disease	9.1 ± 1.8	
Hypertrophic cardiomyopathy	11.3 ± 3.3	
Other	10.9 ± 2.5	
HF duration in months	Rho = − 0.13	0.20
LVEF	R = 0.14	0.18
NYHA functional class		0.85[§]
I	10.4 ± 3.5	
II	10.4 ± 3.6	
III	10.0 ± 3.3	
Hypertension		0.66
Yes	10.2 ± 3.5	
No	10.5 ± 2.3	
Diabetes mellitus		0.24
Yes	10.7 ± 3.4	
No	9.9 ± 3.1	
Renal failure[a]		0.31
Yes	10.0 ± 2.8	
No	10.6 ± 3.6	
Anemia[b]		0.23
Yes	10.8 ± 3.1	
No	10.0 ± 3.3	
Atrial fibrillation/flutter		0.01
Yes	9.3 ± 2.6	
No	11 ± 3.5	
Obesity		0.23
Yes	10.9 ± 3.6	
No	10.1 ± 3.1	
Smoker		0.48
No	10.4 ± 3.3	
Past	10.3 ± 3.3	
Current	8.6 ± 2.4	

Data expressed as mean ± standard deviation, median (25th–75th percentiles), or absolute number (percentage). R and Rho according to Pearson and Spearman correlation, respectively

LVEF left ventricular ejection fraction, *NYHA* New York Heart Association

[§] Scheffe post hoc analyses did not reveal any statistical difference between individual items

[a] eGFR < 60 ml/min/1.73 m^2

[b] Hb of < 12 g/dl in women and < 13 g/dl in men

HF [18]. There remains a need for large, prospective, longitudinal studies to acquire more in-depth insights into the relationship between TL and HF [2]. Herein, we used a novel flow-FISH method to specifically determine TL attrition in circulating monocyte subsets [8].

All previous studies of TL in HF have been cross-sectional in design, and thus have not provided information about possible changes in TL over time or whether any such changes are related to outcomes. Our current report provides the first evidence of accelerated telomere erosion in the monocytes of patients with HF over 1 year. In our study, 96.3% of patients with HF showed shortening of TL at the 1-year follow-up. While this proportion is similar to that observed in general population studies [19], the rate of decline in our cohort is significantly higher than in normal individuals. Indeed, over 50% of the patients exhibited a telomere attrition exceeding − 20% at the 1-year follow-up. The MRC National Survey of Health and Development (NSHD, also known as the 1946 British Birth Cohort) exquisitely reported longitudinal measures of telomere length in a large cohort comprising mainly cardiovascular disease-free participants. In this cohort of healthy individuals, telomere length shortening over ten years was ~ − 2%, as measured by real-time PCR [19].

It is beyond the scope of this study to determine exactly why telomeres shorten faster in patients with HF. However, oxidative stress and inflammation are considered the most important factors contributing to telomeric DNA loss [6], which in turn may favor the development of structural tissue damage. It is postulated that shorter TL may be an irremediable intracellular mechanism facilitating or even causing HF. Indeed, experimental data suggest that telomere shortening partly mediates apoptosis in HF [18]. Furthermore, telomere attrition may lead to increased levels of dysfunctional senescent cells (e.g., circulating monocytes) in tissues and organs, potentially explaining the lower threshold for expressing clinical manifestation of disease [2]. A biomarker's value is largely determined by its capacity to reflect prognosis or change in disease progression; therefore, we followed patients for a mean of 2.3 years. Our data showed no correlation between the rate of telomere attrition and the primary endpoints, suggesting that telomere shortening rate may not be a clinically useful measurement or a viable surrogate marker of disease progression. On the other hand, it is possible that a longer observation period or a larger sample may be required to evaluate these clinical endpoints.

The presented findings also revealed that monocyte TL was associated with atrial fibrillation. Recent investigations of the relationship between TL and atrial fibrillation

Table 4 Cox regression analysis for risk of all-cause death and the composite end-point of all-cause death or heart failure hospitalization based on Telomere length

	All-cause death			Composite endpoint		
	HR	[95% CI]	p value	HR	[95% CI]	p value
Monocytes, whole	1.02	[0.89–1.18]	0.76	1.00	[0.90–1.12]	0.97
$CD14^{++}CD16^{-}$	1.00	[0.87–1.13]	0.94	1.00	[0.91–1.11]	0.93
$CD14^{++}CD16^{+}$	1.03	[0.91–1.18]	0.63	1.02	[0.91–1.13]	0.78
$CD14^{+}CD16^{++}$	1.04	[0.90–1.20]	0.59	0.98	[0.97–1.11]	0.59
Monocytes, % Δ	0.99	[0.95–1.03]	0.57	0.98	[0.95–1.01]	0.24
$CD14^{++}CD16^{-}$, % Δ	1.00	[1.00–1.04]	0.81	0.98	[0.95–1.01]	0.12
$CD14^{++}CD16^{+}$, % Δ	0.99	[0.95–1.03]	0.51	0.98	[0.96–1.01]	0.21
$CD14^{+}CD16^{++}$, % Δ	0.98	[0.94–1.03]	0.44	1.00	[0.97–1.03]	0.75

% Δ available in 54 patients

have produced controversial findings [20]. In the Cardiovascular Health Study (CHS), Roberts et al. [21] compared patients with and without atrial fibrillation, and found no relationship between mean TL and atrial fibrillation. In contrast, Carlquist et al. [22] reported shorter TL in patients with atrial fibrillation, both before and after adjustment for age and other cardiovascular risk factors. However, the exact mechanism leading to shorter TL in patients with atrial fibrillation remains elusive.

Of note, since participants were drawn from the general population that visited a structured HF Clinic located within a tertiary university hospital and our cohort included mainly male patients with ischemic heart disease, further research is needed to determine whether our findings can be generalized to other HF cohorts, such as patients with HF and preserved ejection fraction. As limitations, our sample size or duration of observation (2.3 years) seem to be insufficient to show significant associations between telomere shortening and the tested outcomes. We thus hypothesize that larger follow-up period and many measurement time-points will be crucial to further validate our findings. However, to implement a biomarker in precision medicine, it must show clinical applicability in small numbers of patients, if not in individual subjects.

Conclusions

In summary, the present longitudinal observational study revealed a 22% reduction in TL over 1 year in monocytes from ambulatory patients with HF. Baseline TL and change in TL were not significantly associated with outcomes; therefore, the change in TL is not likely to be a useful biomarker of HF progression.

Additional file

Additional file 1: Table S1. Baseline Characteristics of the Study Participants with one year sample.

Abbreviations

DNA: deoxyribonucleic acid; FITC: fluorescein isothiocyanate; Flow-FISH: flow cytometry-fluorescence in situ hybridization; HF: heart failure; MFI: median fluorescence intensity; PNA: peptide nucleic acid; qPCR: quantitative polymerase chain reaction; TL: telomere length.

Authors' contributions

IT, EE, SR, MF, CGM, PM and MA carried out the experiments; IT, EE, SR, MF, JL and ABG wrote the manuscript; CGM performed figures and artwork; MF, JL and ABG: final approval of the manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests

All authors have read the journal's policy on disclosure of potential conflicts of interest and, accordingly, they state that there are no potential conflicts of interest to declare.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable since the manuscript is entirely original; the tables and figures presented are original for this article and have neither been published nor are currently under consideration for publication by any other journal.

Ethics approval and consent to participate
Each subject gave their written informed consent prior to participation. The study protocol was approved by the Clinical Research Ethics Committee of our institution, was designed in accordance with the principles outlined in the 2013 revision of the Declaration of Helsinki of 1975.

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6. GLOBAL RESUME OF THE RESULTS

In the **first**[80] of the studies of this doctoral thesis was evaluated an innovative technique for the analysis of the relative telomere length with Flow-FISH with the conjugation of a newly developed family of fluorochromes (Brilliant violet) in order to simultaneously identificate the three monocyte subsets. Our cohort included 28 outpatients diagnosed with Heart failure of the multidisciplinary Heart Failure Clinic of the Hospital Germans Trias I Pujol. The majority of the patients were men (71%) with ischemic cardiomyopathy (43%) and mean LVEF of 40%, predominantly in functional NYHA class I and II (79%). The polymer-based BV dyes were heat stable and withstood the stringent conditions of hybridization with no degradation or loss of emission and additionally, the fluorochrome Brilliant Blue 515 showed good thermal resistance. Moreover, the Brilliant violet fluorochrome conjugated antibodies successfully discriminated among the monocyte subsets, with percentages of monocyte subsets measured before and after hybridization very similar when measured with standard fluorochromes (CD15-AlexaFluor647, CD16-BV421, CD86-BV605, and CD14-BV785). The relative telomere length was also measured successfully, respectfully 9.68 ±2.21, 9.76± 2.26, and 9.96± 2.20 for classical, intermediate, and non-classical monocyte subset.

In this way, we created and applied a complete innovating technology for assessing the monocytes and their telomeres.

In the **second** study[76] we analysed 400 consecutively ambulatory patients from the same Heart failure clinic, most of who were referred from the Departments of Cardiology and Internal medicine, with similar baseline characteristics as in the study mentioned above: predominantly middle aged male (73%) with reduced LVEF (<40% in 45%), ischaemic aetiology (46%) and in NYHA II. During a mean follow-up of 2.6± 0.9 years, 107 patients died, 99 had a HF-related hospitalization and 160 suffered the composite end-point of all-cause death or HF-related hospitalization. No correlation between subset percentage or subset number of cells/μL and NYHA functional class, LVEF or estimated glomerular filtration rate was found, except for the percentage of intermediate monocyte subset which showed a weak inverse correlation with

LVEF. When the monocyte subset distribution was assessed according to the different aetiologies of HF, globally there were no statistically significant differences when percentages of subsets were considered, but a significant difference was observed in the non-classic subset when number of cells was measured (p = 0.04). Significant differences were found between alive and deceased patients at the end of the study for the non-classical monocyte (CD14+/CD16++) subset considered as percentage, and in intermediate subset when absolute cell count (number of cells/ μ L) was considered.

When considering percentages of monocyte subsets, non-classical monocyte subset showed protective significant association with all-cause death and the composite end-point in the univariable analysis. Nevertheless, in the multivariable analyses, it did not remain related any of these end-points. In contrast, when considering absolute cell count, the intermediate (CD14++/CD16+) monocyte subset showed detrimental association associated with an increase of all-cause death (HR 1.25 [95% CI 1,02-1.52], p = 0.03), and the composite end-point HR 1.20 [95% CI 1,03-1.40], p = 0.02) and was borderline associated with Heart failure-related hospitalization, while the non-classical (CD14+/CD16++) subset showed protective association with all-cause death and borderline significance with the composite end-point in the univariable analysis. The quantitative determination of the absolute cell count of each monocyte subset expressed by U/mL was superior from the prognostic point of view than the percentage of these monocyte subsets in outpatients with HF. Classical subset was independently associated with HF-related hospitalization and the composite end-point and non-classical subset with all-cause mortality.

In the **third** study[81] was investigated the relative telomere length and its attrition during a follow up of a year in a cohort of 101 ambulatory patients with heart failure, recruited from the same clinic. The patients shared baseline and clinical characteristics with the two previous

studies, described in this thesis: male, ischemic aetiology, NYHA functional class II or III and were treated following contemporary guidelines.

The three different monocyte subsets were assessed and their respective telomere length was measured. There was a high correlation between the telomere length and the different monocyte subsets: CD14++CD16+ vs. CD14++CD16−, $R = 0.95$, $p < 0.001$; CD14++CD16+ vs.CD14+CD16++, $R = 0.90$, $p < 0.001$; and CD14++CD16−vs. CD14+CD16++, $R = 0.89$, $p < 0.001$. Within the subgroup of patients, for whom blood samples were available at baseline and at one year follow up, we found a statistically significant (~ 22%) attrition of mean monocyte telomere length (11.1 ± 3.3 vs. 8.3 ± 2.1, $p < 0.00$), a reduction which occurred in 96.3% of the patients (ranging from − 3.1 to − 66.7%); in 28 of these patients (51.9%) monocyte telomere length reduction was ≥ − 20%. Telomere length at baseline, telomere length at the 1-year follow-up, and the change in telomere length over 1 year did not significantly differ between the monocyte subsets (all p values > 0.1) nor were any difference between genders or age, sex, ischemic aetiology, presence of diabetes mellitus or Heart failure duration (< 48 months vs. longer). Over the mean follow-up of 2.3 ± 0.6 years, 17 patients died, 17 required hospital admission due to Heart failure, and 29 suffered the composite end-point of death or Heart failure hospitalization. Nevertheless, these outcomes were not associated with telomere length at baseline or with the change over a year.

7. GLOBAL DISCUSSION OF THE RESULTS

7.1. The use of Fluorochromes from the Brilliant violet family for the simultaneous analysis of telomere length and identification of the monocyte subsets resulted to be an innovating, accurate and cost-effective method

Necessity of new techniques for assessment of the monocyte subsets and their telomere

The established techniques for analysis of the telomere length have many inconvenients such as fluorescence intensity, suboptimal analytical sensitivity, conjugation to heat stable fluorochromes; it is required large sample amounts and the obtained results have often different samples variability. The flow-FISH methodology has demonstrated to be a very useful and valuable technique for the analysis of cell subpopulations with the option to detect the expression of specific surface antigens in the cells or cells subsets. However, it presents some difficulties when combined with analysis of the telomere length[77,78,82]. It is challenging to perform a combined analysis of cell phenotyping and telomere length due to the harsh conditions for the hybridization of the telomere probe (in concrete, intense fixation and incubation at 82°C).

Another critical step, often performed in the flow-FISH measurement of telomere length to counteract the loss of fluorescence of the conventional fluorochromes, is the expensive and relatively difficult cell purification. As alternatives to this process, some authors have described immunophenotyping for cell subsets identification and then have applied calculations for the telomere length or use of heat stable fluorochromes instead of convenient once. Other limitations in standard protocols for telomere length and cell characterization is the small number of cells obtained for telomere length analysis and the autofluorescence during the analytical process[83].

On one hand, such analysis implicate that the probe has to be exposed to the harsh conditions of intense fixation and incubation up to 82°C; on the other hand, to resist these conditions could be a problem for some of the most frequent used fluorochromes. Fluorescent probes are divided into three main groups of families: large protein-based molecules (phycobiliproteins), inorganic fluorescent nanocrystals (quantum dots) and small organic dyes (fluorescein). Many of these fluorochromes lack in brightness, have a spectral overlap, different stability or applicability to different techniques, inclusive cell staining[84]. Moreover, subset purification from conjugates could difficult the analysis, being complicated, inefficient, expensive and time consuming, because of loss of fluorescence when non-heat stable organic fluorochromes are applied during the immunophenotyping step.

The family of Brilliant violet fluorochromes

In our study we investigated if a newly developed Brilliant Violet dyes are valuable heat stable alternatives compared to conventional organic or protein-based fluorochromes in flow-FISH. We tested the Brilliant Violet dyes to specify and simultaneously assess the distribution and telomere attrition of monocyte subsets in blood of patients with heart failure.

The fluorochromes from the Brilliant Violet family are characterized with brighter fluorescence than standard fluorescent dyes (as the most frequent used fluorochromes: allohycocyanin, APC and phycoerythrin (PE).[84] These dyes have unique optical characteristics and very distinguish structure. About 1000 fluorescent monomeric subunits per macromolecule act cooperatively, resulting in emission of light with molecular extinction coefficients and high quantum efficiency.[85,86] These fluorochromes are easily and reliably conjugated to antibodies, they have high solubility and little non-specific binding as well as high intrinsic brightness[84,85], making them suitable for intracellular staining. Another advantage of the Brilliant Violet dyes includes synthetic modifications of their backbone structure to span the full range of the visible spectrum and spectral compatibility with each other for multicolour simultaneous labelling. Quantum

dots (QD), an inorganic semiconductor nanocrystal, is read in regions of spectrum with low autofluorescence and a thermally stable under hybridization[87,88] unfortunately it has significant overlaps with other channels due to multilaser excitation and difficult conjugation to antibodies, which makes it application limited. Other dyes like cyanines and Alexa Fluor are also thermally stable[87,88], but have the disadvantages of few appropriate reagents, the difficult conjugation to antibodies, consequently they are scarcely used for detection of cell surface antigens. In comparison, fluorochroms of the Brilliant Violet family allowed better resolution of cell surface markers[84].

Their polymeric structure and their benefit in multicolour staining panels suggest that the Brilliant Violet family of dyes may be successful alternatives to the brightest fluorochromes[86] commonly used for immunophenotyping purposes, such as phycoerythrin and allophycocyanin. Moreover, their use enables the violet laser for polychromatic excitations. Brilliant Violet dyes are more thermally stable than most low molecular weight fluorochromes, which lose their fluorescence under the intense fixation and high temperature (82 °C) required for DNA denaturation and hybridization of the fluorescent telomere probe. Based on the qualities described above, we hypothesized that Brilliant Violet, as well as the new polymer-based dye Brilliant Blue BB515, would be appropriate alternatives to conventional fluorochromes in assays designed to simultaneously identify specific cell subpopulations (immunophenotyping) and hybridize probes to telomeres in situ. We also tested stabilization of the fluorochrome–antibody complex with the amine-to-amine cross-linker bis sulfosuccinumidyl suberate. This cross-linker is homobifunctional, water-soluble, noncleavable, and membrane impermeable; all of these properties are essential for Brilliant Violet -derived prehybridization fluorescence emission[89]. To evaluate which Brilliant Violet dyes perform best in the detection of CD14, CD16, and CD86 expression in monocyte subsets and telomere length calculation, we measured

the levels of cellular autofluorescence induced by treatment and the spectral overlap of DNA dyes required for the identification of cell cycle phases.

In our laboratory, propidium iodid (PI) staining decreases sensitivity in V560–V660 channels and this was taken into account. Moreover, fluorochrome selection had to be optimal relative to expected antigen densities and dye efficiencies[90].Thus, in the present study, CD16-Brilliant Violet 421, CD86-Brilliant Violet 605, and CD14-Brilliant Violet 786 were chosen for scrutiny.

Brilliant violet fluorochromes and flow-FISH analysis

We focus on the identification of an innovating method to asses at a time the monocyte subset distribution and the telomere length. In this context, to find alternative fluorochromes to be used in multicolour flow-FISH and resist the DNA denaturalization and at a time differentiate among the monocyte subsets with correct quantification of their relative telomere lengths, was of upmost importance. Due to the difficulty to separate monocytes with CD14/CD16 antigens from other CD16+ leucocytes as neutrophils, it was necessary to use a fluorochrome, which wouldn't lose brightness or its capacity for differentiation during the fixations steps. For the discrimination monocytes and neutrophils we used additional cell surface antigens, such as an exclusion marker for neutrophils (CD15).

Despite of these conditions, the Brilliant Violet dyes, as well as our new methodology, showed to be effective, three times less expensive and accurate in assessing monocyte subsets and telomere lengths in heart failure patients.

7.2. Distribution and levels of the Monocyte subsets, assessed in number of cells/μ and not in percentages, were independently associated to adverse events in heart failure patients

Distribution and levels of the Monocyte subsets in our cohort of Heart failure patients

Monocytes, referred as well as mononuclear phagocytes or monocytes/macrophages, have multifunctional with roles in homeostasis, defence of the immune system, and tissue repair; depending on the subset, they can have both damage and repair functions in different cardiovascular diseases[35].

In human peripheral blood the three monocyte subsets coexist in different proportion (about 90% the CD14+ rich subsets and 10% the CD16+ rich subset, which means approximately 83.5% from the classical subset, 11-13% non-classical and 3.5-5% intermediate subset depending on the measurement,[47] however this proportion is highly variable and it can be influenced from the specific needs of the organism in concrete moment of time, being difficult to separate the non-classical from the intermediate subset. This explains why in other review[91] it was observed that in healthy individuals the distribution and levels of the monocyte subsets were different, with significant prevalence of the classical (CD14++/CD16-) monocyte subset (80.1+-7%) in comparison to the other two subsets: the intermediate, CD14++/CD16+(3.71+-2.0%) and non-classical, CD14+/CD16++(6.2+-2.8%). These proportions are also highly variable in the course of a concrete disease and may be related to pathogenesis and the specific functions of the monocytes, as reported in the publication of Ziegler, where the intermediate subset experienced significant variation in the course of a sepsis infection[35,47].

Another study described that a significant expansion of the intermediate subset was observed in blood during ST-elevation myocardial infarction, which correlated with troponin elevation and left ventricular function[92]. In our cohort of 400 outpatients with heart failure we observed that the percentages of the monocyte subsets have different distribution in comparison to healthy controls[91] with significant expansion of the intermediate subset (42+- 17.2%); still the classical subset was the predominant subset with 50.0+-17.2% and less frequent was the non-classical subset with 8.1+-4.0%. To better understand the pathophysiological mechanism for this data, it is from crucial importance to have in mind the baseline characteristics of our patients who were predominantly elderly males, with chronical heart failure from ischemic aetiology, both of which have been associated with chronical state of inflammation, a situation that predisposes to the expansion of the intermediate subset.

Expansion of the intermediate monocyte subset

Although this monocyte subset is small in healthy individuals (8-10%), an expansion of the intermediate subset has been described also in other clinical conditions (as described previously in this thesis) such as diabetes, chronic vascular and endothelial damage and atherosclerosis,[95,97-98] chronic inflammatory disorders like rheumatoid arthritis[96,104-106.] Being rich in IL1 (pro-inflammatory cytokine), together with other pro-inflammatory cytokines, the intermediate subset can inclusive produce heart damage[45]and it has been demonstrated that this subset could predict cardiovascular events in dialysis patients[99].

The expansion of the intermediate subset in our patients could be explained with its unique characteristics[34,36,47], sharing similar qualities with both the classical and non-classical subsets[92-94]. In concrete, its elevated levels may be interpreted by its pro-inflammatory nature in the permanent stage of inflammation, observed in Heart failure[101,102]. Another interesting point, discussed in the article of C. Baristone et al.[48] is the elevation of the monocyte ACE (angiotensin converting enzyme) by the intermediate subset in heart failure patients, with

consequently permanent activation of the renin-angiotensin system, which could explain the pro-inflammatory effect of these monocytes and the state of permanent systemic inflammatory condition in chronic heart failure. Even, it was suggested that by influencing the inflammation process in heart failure, agents that inhibit the expansion of the intermediate subset might be used in order to prevent myocardial damage and dysfunction. Some limitations of the study of Baristone were the small number of patients included and the exclusive inclusion of patients with reduce LVEF, not considering patients, diagnosed with heart failure but with preserved EF.

The intermediate subset produces higher levels of pro-inflammatory cytokines (IL-1beta, IL-10, TNF alpha and oxygen radicals)[95,] it is describe to response to lipopolysaccharide (LPS) and zymosan[47.] Zawada et al. and Grage-Griebenow identified the intermediate subset as the subset with the highest capacity to induce superatigen –mediated T-cell proliferation and it possesses a unique a phagocytic activity and capacity for antigen presentation and T-cell interaction[92-94]. Other studies have confirmed the importance of the inflammation and immunological process in the pathophysiology and progression of heart failure[101,102]. The majority of the monocytes (especially the classical subset, CD14+CD16-) participate in the resolution of inflammation, while, in comparison, the minor subpopulation of CD16+ promote the inflammation process and have antimicrobial ability. Since the monocytes are the precursors of the macrophages in the tissues, the macrophages that are generated from CD16+ monocytes have higher phagocytic activity in comparison to macrophages proceeding from the classical subset[100].

The intermediate subset, as written above, has been investigated in other studies[107,108] and it has been linked to diseases with increase infection/inflammation processes like sepsis. This subset was increased as well in patients both with acute and chronic heart failure[45] in comparison to healthy individuals, as was observed in our cohort of chronic heart failure patients. It is important to outline that some of our patients have not only heart failure but also other chronic

diseases (cancer) and cardiovascular risk factors (diabetes, hypercholesterinaemia, high blood pressure), which may have aggravated their clinical condition where the maintenance of cardiac integrity through removal of damage of dead cells is crucial.

The intermediate monocyte subset provides information on increased risk of all-cause death and the composite end-point in heart failure patients

In general, there are many studies about the distribution of the monocytes subsets in different human disorders as described above, but very few are focused on the clinical importance and significance in patients with heart failure[33,73].

In our study we investigated the distribution and levels of the different monocyte subsets and if they were related to adverse outcomes: mortality and hospitalizations due to heart failure or both.

As described in the study of the ST-elevation Myocardial Infarction[109], in the healing process of a vascular/endothelial damage different stages are present, in which different cell types and substances interfere. An initial migration of pro-inflammatory cells such as the intermediate subsets is essential for the first step of the early wound healing, but their persistence in the damaged tissue could aggravate, even more, produce extra damage to the myocardial tissue, which may explain the poorer prognosis of the heart failure patients with higher intermediate monocytes. In the study of Zeng et al. as well as in Rogacev KS et al. the intermediate subset was considered a risk factor post-ST elevation myocardial infarction for adverse outcomes and cardiovascular events[40,109]. In our study, we also found a significant difference for the intermediate subset when we measured the levels in number of cells/µL. Remarkably, although patients who died had worse clinical characteristics (higher age, worse NYHA functional class, worse LVEF, higher NT-proBNP, worse renal function) the intermediate subset was independently associated with all-cause death and the composite end-point in the multivariable analyses. In patients with acute heart failure, where the pro-inflammatory condition is much

more accentuated in comparison to our ambulatory patients with chronic heart failure, Wrigley et al. also reported that the intermediate subset experienced an expansion in acute heart failure patients and was related with poorer prognosis[73].

Absolute cell count vs percentages of the monocyte subsets as predictor factor for adverse events in Heart failure

Absolute cell counts have been shown to be a major tool for laboratory diagnosis for a variety of pathological conditions, including CD4+ T-cells for Acquired Immune Deficiency Syndrome and haematopoietic progenitor cells expressing the possibility of selection bias represents a potential limitation of the study.

An additional relevant finding in the present study is the assessment and prognostic value of monocyte subset absolute cell count, beyond the classical approach of subset percentages. Monocyte cell count (defined as number of cells/μL) has been assessed in previous studies of healthy patients[91]. We found that the absolute count was markedly superior to the percentage distribution of each subset when considering the prognostic role of the different monocyte subsets. The presented findings show that absolute cell count of monocyte subsets was preferred over monocyte percentage for prognosis stratification for outpatients with Heart failure. We cannot completely understand why exactly the absolute cell count and not the percentages showed this association, but possible bias in the measurements or procession of the samples or in the chosen statistic method could have influenced the results.

7.3. Telomere attrition was observed in the majority of the patients with heart failure, nevertheless, this reduction wasn't related to adverse outcomes

Telomere attrition in chronic heart failure

The predisposition and the development of heart failure, as other human conditions, have been associated with the aging process of the organism[48-50], due to the pathophysiological underling mechanisms and age-associate changes on cell level: the permanent exposure to oxidative stress radicals, advance glycation endproducts and apoptosis. With time, the relative length of telomeres shortens, fact which, as described above, is a phenomenon related to the development of cardiovascular diseases and heart failure.

The telomere length is considered by many authors as a "biological clock"[110,111], but it is only one parameter, which determine the integrity of the telomere complex. Many human disorders are related to alterations in the telomerase enzyme[51]. Decreased activity was described and extremely short telomeres were reported in dyskeratosis congenita, multi-system disorders, pulmonary fibrosis, premature aging and bone marrow insufficiency; on the contrary, increased activity is observed in uncontrollable growth and neoplasia[51].

Still, little is known on the telomere attrition and telomere length in patients with chronic heart failure[62]. Even less investigated is the telomere length change and its significance in monocyte cells of patients with heart failure. In our study, we investigated a cohort of one hundred ambulatory heart failure patients and followed them during at least one year to better understand the dynamics of telomere attrition in circulating monocytes. Preliminary data from cross-sectional studies reveal shorter telomeres in Heart failure patients compared to healthy age- and gender-balanced controls, based on measurements in circulating leukocytes using a conventional qPCR method[52]. Moreover, telomere length is reportedly associated with the

severity of Heart failure symptoms and outcome[52,56], and with worse renal function in subjects with Heart failure, which is a powerful predictor of outcome[46,59,64]. We apply a new protocol and methodology[80] for simultaneously assess the telomere length with flow-FISH analysis and investigated the relationship between the telomere length and adverse events (mortality and hospitalizations due to heart failure decompensation). We were able to specifically determine the telomere length attrition in the different circulating monocyte subsets and we observed an accelerated telomere erosion in the monocytes of our patients with heart failure over 1 year. In our study, 96.3% of patients with heart failure showed shortening of telomere length at the 1-year follow-up. While this proportion is similar to that observed in general population studies[112], the rate of decline in our cohort is significantly higher than in normal individuals. Our date showed over 50% of the patients exhibited a telomere attrition exceeding 20% at the 1-year follow-up. The MRC National Survey of Health and Development (NSHD, also known as the 1946 British Birth Cohort) exquisitely reported longitudinal measures of telomere length in a large cohort comprising mainly cardiovascular disease-free participants. In this cohort of healthy individuals, telomere length shortening over ten years was approximately 2%, as measured by real-time PCR. It is not very clear the reason exactly why telomeres shorten faster in patients with heart failure. However, oxidative stress and inflammation are considered the most important factors contributing to telomere DNA loss[113-115], which in turn may favour the development of structural tissue damage. It is postulated that shorter telomere length may be an irremediable intracellular mechanism facilitating or even causing heart failure. Indeed, experimental data suggest that telomere shortening partly mediates apoptosis in heart failure[116]. Furthermore, telomere attrition may lead to increased levels of dysfunctional senescent cells (e.g., circulating monocytes) in tissues and organs, potentially explaining the lower threshold for expressing clinical manifestation of disease[48].

Telomere length and its value as a predictor factor in patients with chronic heart failure

A biomarker's value is largely determined by its capacity to reflect prognosis or change in disease progression. We followed our patients for a mean of 2.3 years. Our data showed no correlation between the rate of telomere attrition and the primary endpoints, suggesting that telomere shortening rate may not be a clinically useful measurement or a viable surrogate marker of disease progression. However, it is possible that a longer observation period or a larger sample may be required to evaluate these clinical endpoints.

The presented findings also revealed that monocyte telomere length was associated with atrial fibrillation. Recent investigations of the relationship between telomere length and atrial fibrillation have produced controversial findings[117]. In the Cardiovascular Health Study (CHS), Roberts et al.[118] compared patients with and without atrial fibrillation, and found no relationship between mean telomere length and atrial fibrillation. In contrast, Carlquist et al.[119] reported shorter telomere length in patients with atrial fibrillation, both before and after adjustment for age and other cardiovascular risk factors. However, the exact mechanism leading to shorter telomere length in patients with atrial fibrillation remains elusive and we can neither recommend the use of the telomere length or attrition as a commonly used predictor factor for prognosis in Heart failure nor in atrial fibrillation in the general population.

7.4. Limitations of the project

1. The possibility of selection bias represents a potential limitation of the study. The subjects reported here were drawn from a general population that visited our tertiary hospital heart failure Unit. The majority had been admitted to the hospital in previous years and the cohort included primarily male patients with ischemic heart disease as the main cause of heart failure and depressed left ventricular ejection fraction. Given the selected characteristics of the patients in the studied cohorts as described above, further research is needed to determine whether our findings can be generalized to other heart failure cohorts, such as patients with heart failure and preserved ejection fraction or older patients.
2. Another important issue is that we have analysed only one blood sample per patient, and cannot comment on the prognostic value of serial determinations. Indeed, although blood samples were obtained in routine ambulatory visits, we cannot discard that in some isolated patient it could have been obtained after a relatively near inciting incident (i.e. exacerbation) which could have mobilized the cells of interest.
3. We did not have data on other inflammatory cytokines such as TNF, IL6, or IL1b, which might could yield insights into the mechanistic role of differential monocyte distribution.
4. The sample size of our study and the duration of observation (2.3years) might be insufficient to show significant associations between telomere shortening and the tested outcomes. A larger follow-up period and many measurement time-points might help to further validate our findings. However, to implement a biomarker in precision medicine, it must show clinical applicability in small numbers of patients, if not in individual subjects, that's why the telomere shortening was not useful as a predictor factor in this context.

8. CONCLUSIONS

1. The classical (CD14++/CD16±) monocyte subset was decreased and the intermediate (CD14++/CD16+) monocyte subset was increased in patients with heart failure compared to reported controls. The quantification of the absolute cell count of each monocyte subset (number of cells/μL) showed a superior prognostic value for the studied cohort of patients, compared to the performance of monocyte subset analysis by percentages. The intermediate subset was independently associated with all-cause death and the composite end-point of all-cause death or heart failure hospitalization, in multivariable analyses.

2. Brilliant Violet fluorchromes completely tolerated the harsh conditions for the DNA denaturalization and simultaneously provided accurate identification of the monocyte subsets and the telomere length. Therefore, they can facilitate accurate, specific, relatively inexpensive and faster measurement of circulating monocyte subsets and their respective telomere length in the context of heart failure.

3. Our study revealed a 22% reduction in telomere length over 1 year in monocytes from outpatients with heart failure. Nevertheless, baseline telomere length and change in telomere length were not significantly associated with outcomes in ambulatory heart failure patients. Therefore, the change in telomere length is not likely to be a useful biomarker of heart failure progression.

9. FUTURE LINES OF RESEARCH

1. This project is pioneer in the study of the levels and function of the different monocyte subsets in patients with heart failure. We have centred our investigation in chronic heart failure, but research in acute heart failure might be also very interesting and valuable, taking into consideration the inflammatory environment of such acute situation. This is a very important field to explore.
2. The development of our new methodology for the assessment of the monocyte subsets and their telomeres is clinically relevant and can be expanded to other diseases or pathologic situations, being able to have direct consequences for future prognostic assessment and maybe therapeutic interventions in many patients.
3. The consequences of increased telomere attrition in the longer term in heart failure patients are an open question, which might originate new futures investigations.
4. The monocytes and monocyte subsets could be used not only as a predictor factor but also might be taken into consideration as part of an immuno-modulation therapy in the future for the heart failure patients.

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