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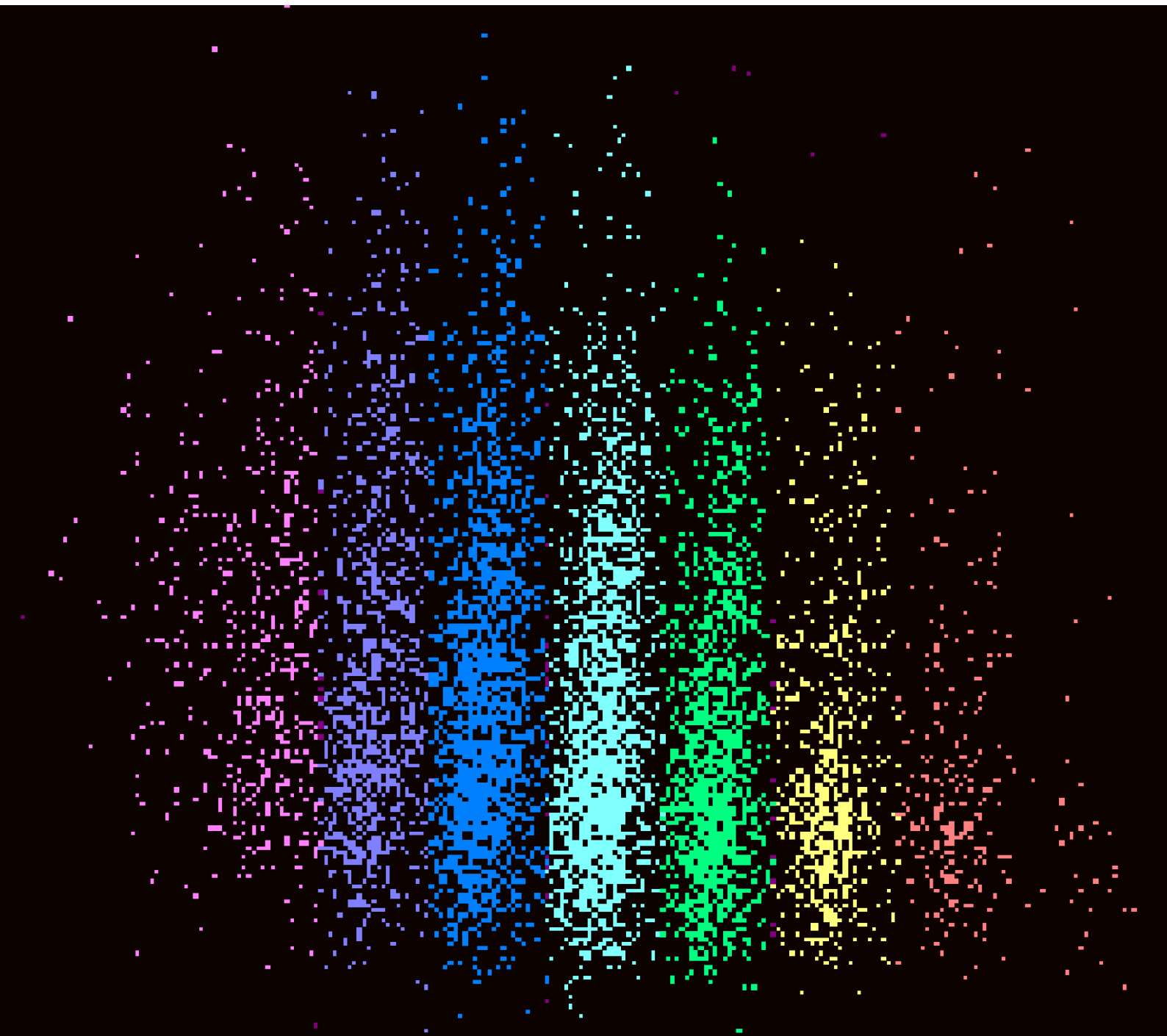

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Contents

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Cardiovascular risk prediction- now and the future

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ABSTRACT

Current cardiovascular risk estimation systems that estimate 10-year risk based on cohort studies starting at around age 40 have probably reached their limits based on current methods.
The challenges are to develop new systems that will permit personalised risk estimation earlier in life with better estimates of true lifetime risk and likely treatment benefits. We outline approaches to address these issues.
Disclosures: I have no conflicts of interest, intellectual or financial.
Acknowledgements: This review is based in part upon the work of the European Society of Cardiology's Cardiovascular Risk Collaboration, of which the author was a founder and co-Chair. It is a pleasure to acknowledge Panos Vardas, Adam Timmis, Victor Aboyans, Emanuele di Agelantonio, Dirk De Bacquer, Brian Ference, Frank Visseren, Bill McEvoy and the staff of the ESC's European Heart Health Institute.
Terminology: It is perhaps pedantic to observe that, although the term 'cardiovascular risk prediction' has become embedded in the cardiological literature, what we in fact do is to estimate the risk to allow a prediction of the likelihood of a future clinical event. The term 'screening' strictly applies to testing to assess the likelihood of disease. 'Health risk assessment' is a wider term that includes demographics, social factors, lifestyle and assessment of risk factors.

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Keywords
Cardiovascular risk estimation; lifetime risk

Why assess cardiovascular disease (CVD) risk?

Cardiovascular risk assessment is used to guide management decisions. In general, the higher the risk, the more intense will be the preventive efforts required.

In most people, the risk of a future atherosclerotic CVD event is the product of the combined effect of a number of risk factors such as hyperlipidaemia, hypertension, smoking and diabetes. The clinical estimation of the effect of such combinations is unreliable, which is the rationale for risk scoring systems [1].

When to assess CVD risk

Current risk estimation systems are based on cohort studies that started at about age 40 and so estimate risk from then on. This misses 40 years of exposure to risk, in addition to in-utero risk. The future, discussed below, is clearly to develop systems that can estimate risk much earlier in life.

Risk evaluation may be opportunistic (when a person presents for another reason) or systematic, either population wide of in defined groups with known risk factors such as smoking or diabetes. Population wide risk assessment allows improvement in risk factors but it has been difficult to demonstrate improved outcomes [2], and hence cost-effectiveness is uncertain. Many countries prefer a combination of opportunistic evaluation and evaluation in those with known risk factors.

How to assess risk

Both the 2021 ESC Prevention Guidelines [2] and The 2019 ESC/EAS Guidelines for the management of dyslipidaemias [3] define categories of risk. In general the latter adopts a simpler approach but both agree that subjects with established CVD have declared themselves to be at very high risk and intensive and immediate risk factor advice is advised.

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In *apparently health persons,* The European Society of Cardiology 2021 Guidelines on the Prevention of Cardiovascular Disease in Clinical Practice [2] recommend the use of SCORE2 [4] or, in persons over 70 years, SCORE2-OP [5] for risk assessment. These tools are calibrated for four risk regions of Europe and can be re-calibrated for other countries. HeartScore is a simple, interactive online calculator that facilitates the use of SCORE (www.heartscore.org)

In those at intermediate risk, screening for asymptomatic disease, for example through coronary artery calcium scoring may help to re-classify risk [2].

In America, use of the Pooled Cohort equation is recommended [6], more recently supplemented by the PREVENT calculator [7].

Limitations of current risk estimation systems

Current risk estimation systems are derived from cohorts studies, most of which started at about age 40, in other words after many years of exposure to risk. Strictly speaking, they apply only to the population from which they were derived. They may work well in other similar populations, or can be re-calibrated for others [4, 5], but the problem remains that the risk estimates apply to groups rather than individuals.

Current techniques such as Cox derive beta-coefficients that are essentially multipliers and cannot easily estimate complex interaction effects within different combinations of risk factors. Further, risk estimates are dominated by the effect of age, especially when risk is expressed over 10 years. Current estimates of lifetime risk also start too late, usually around age 40.

The impact of genetic factors has been underestimated. While polymorphisms affecting risk may have a seemingly small impact on 5-10 year risk, their impact on true lifetime risk, from birth on, may be much greater than is generally appreciated [8].

Can we see the future?

Ideally, one would like to be able to:

- allow better for the dominance of age in risk estimation
- estimate true lifetime risk from early in life
- approach more individualised estimates of risk
- make more precise estimates of treatment benefits
- explore integrating in-utero determinants of risk

Ference and others [8, 9] have pointed out that Mendelian randomisation studies suggest the impact of polymorphisms on risk has been greatly underestimated, given that they function from birth on. These effects may be direct or, probably more importantly, through their effect on determining the rate of rise of risk factors such as LDL cholesterol and blood pressure.

This has led to a suggestion to move from 10-year risk to an exposure time model in which risk is expressed as mmol/years of LDL cholesterol, mmHg/years of blood pressure or indeed years of exposure to total risk. Such an approach integrates the rate of rise of risk with time which is likely to parallel the development of atherosclerosis. Thus, given several measurements of risk over several years in younger persons, it should be possible to give a personalised estimate of risk much earlier in life than is currently possible to allow true preventive action early in life. This Mendelian Randomisation-based approach can also permit more precise and logical estimates of likely treatment benefits.

The exposure time approach may be summarised as depicted in **Figure 1**.

Vardas has commented on the transition from ancient medicine through modern medicine to what he terms metaclinical medicine [10]. The latter includes, inter alia, artificial intelligence (A-I) and decision-making models. AI is indeed necessary for the approaches summarised above and the need will grow, necessitating dialogue between medical statistics and A-I [11].

Generative A-I can be used to develop risk estimation systems. Starting with existing large data sets, A-I is used to examine patterns and interactions faster and more efficiently than can be done with conventional statistics. A subset is used for machine learning followed by deep learning such as layered neural networks and generative A-I to produce new content.

Alas, it is of course not that simple [11]. Issues include:

- Data quality. No system can allow for poor quality or non-representative data
- Conclusions based on inadequate data may be re-enforced- the 'self-fulfilling prophecy'
- Arising, results may not seem justified by expectations based on the training set- so-called A-I 'hallucination'
- Conventional statistics use clearly verifiable methods. The deeper one goes into machine learning, the more opaque the process becomes
- 'Data-set shift' [12], in which there is a mis-match between the machine-learning model's training data and the results when the model is applied. This is of course not necessarily a fault of the process if it is applied to a very different population

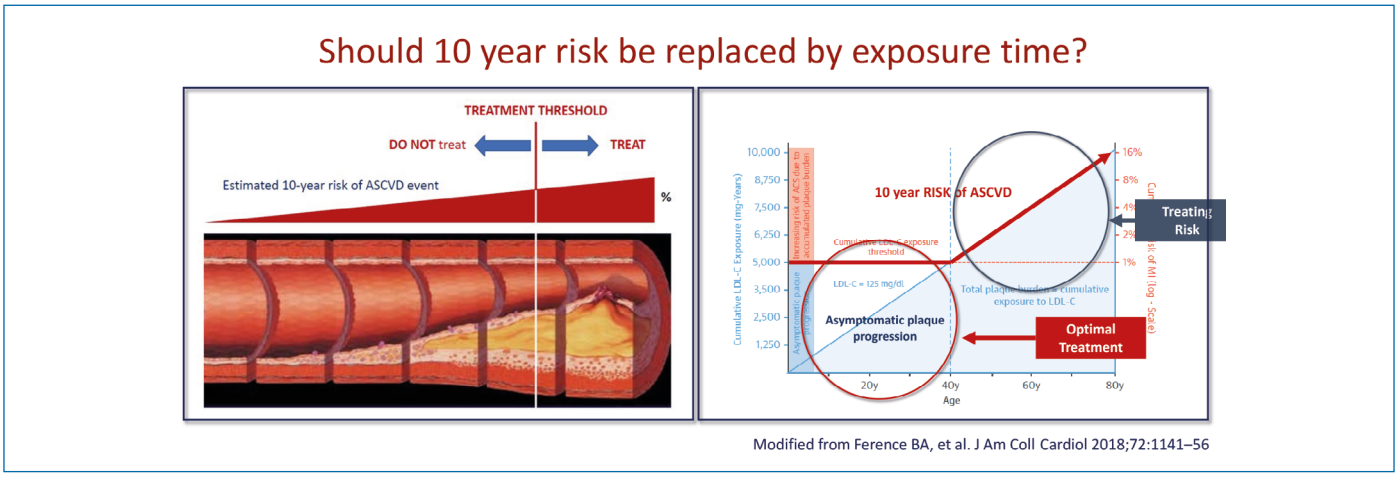

Figure 1 | The exposure time model compared with estimation 10 year risk at, say age 40 -modified from the concepts expressed in [9].

Will these advances produce better outcomes? Ference (personal communications and late-breaking session presentations at the European Society of Cardiology and American Heart Association and American conferences) provides compelling arguments. Yet it is hard to envisage how to design a randomised controlled trial to compare usual care with conventional risk estimation and with the A-I based exposure time approach. The clinician is advised to simply see if a risk estimate, based on whatever estimation process, is plausible.

A comparison of machine learning and conventional risk estimation [13] found in favour of machine learning by a modest amount but with substantial caveats-

"In this systematic review and meta-analysis, ML algorithms were found to be superior to traditional risk equations on comparison of C-statistics in the pooled meta-analysis of 11 studies.

However, findings need to be interpreted with caution as the quality of studies was sub-optimal- with all studies performed on retrospective cohorts, half of the studies providing no comparative calibration metrics, and only three with external validation. In addition, most studies were assessed to have a high risk of bias".

Finally, should determinants of risk in utero [14] be incorporated into a single approach to risk estimation? Those with low birth weight may benefit from early assessment and management of risk. A fully integrated approach to risk from conception through childhood and into adult life would seem logical.

Conclusion

Risk estimation had become rather static. We now enter an exciting new era of risk estimation based on A-I supported risk estimation, Mendelian randomization and risk expressed as exposure time that has the potential to permit personalised risk estimation early in life with better estimates of likely treatment effects.

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Role of nuclear medicine assessing patients with suspected coronary artery disease

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ABSTRACT

Nuclear medicine is a critical component in the field of cardiology as it provides diagnostic and prognostic insights that are essential for the effective management of heart disease.

Both single photon emission computed tomography (SPECT) and positron emission tomography (PET) play a significant role in assessing the likelihood of ischemic heart disease based on pre-test probabilities. Both SPECT and PET should be integrated into the clinical pathway according to the patient's individual risk profile, symptoms, and initial test results. The guidelines recommend using these imaging modalities to refine risk stratification, particularly in intermediate-risk patients, and to guide further invasive diagnostic or therapeutic procedures based on the imaging findings.

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Keywords
Coronary artery disease; single photon emission computed tomography; positron emission tomography; non-invasive tests

Initial evaluation of patients with suspected ischemic heart disease

According to the 2019 European Society of Cardiology (ESC) guidelines on chronic coronary syndromes, a patient with suspected ischemic heart disease, commonly referred to as coronary artery disease (CAD), is typically identified based on risk factors, clinical presentation, and initial non-invasive evaluation [1].

The presence of risk factors such as hypertension, dyslipidemia, diabetes, smoking, a family history of early coronary artery disease, and obesity increases the likelihood of coronary artery disease. Symptoms can be summarized as dyspnea, typical angina pectoris characterized by chest pain or discomfort that occurs with exertion or emotional stress and is relieved by rest or nitroglycerin, atypical angina and non-anginal chest pain when not all the criteria for typical angina are met.

A detailed initial assessment, including medical history, physical examination, and diagnostic tests like an electrocardiogram (ECG), is used to define the pre-test probability of CAD, based on age, sex, and the nature of chest symptoms.

Subsequent management can range from lifestyle modifications and medical treatment for low-risk patients to more aggressive interventions such as revascularization for those at high risk.

Noninvasive diagnostic evaluation

Non-invasive tests to assess ischemia include several techniques, such as exercise testing, stress echocardiography, myocardial perfusion imaging by nuclear imaging with Single Photon Emission Computed Tomography (SPECT) or Positron Emission Tomography (PET), and cardiac magnetic resonance (CMR). These tests help to detect myocardial ischemia and evaluate the need for further invasive investigations such as coronary angiography.

Each non-invasive diagnostic test has a particular range of clinical likelihood of obstructive CAD where the usefulness of its application

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is maximal [1]. Given the clinical likelihood of obstructive CAD and the likelihood ratio of a particular test, one can assess the post-test probability of obstructive CAD after performing such a test [1]. Using this approach, one can estimate the optimal ranges of clinical likelihood for each test, in which they can reclassify patients from intermediate to either low or high post-test probability of CAD [2].

Patients can be categorized as having low, intermediate, and high pre-test probability of having ischemic heart disease and the choice of diagnostic tests is guided by these categories [1]:

- Low Pre-test Probability (<15%): For these patients, non-invasive testing might often be unnecessary, and routine testing is not recommended as it could lead to false positives and unnecessary further invasive procedures.
- Intermediate Pre-test Probability (15-85%): This group benefits the most from non-invasive imaging tests like stress echocardiography, SPECT and PET. SPECT is commonly used due to its availability and efficacy in detecting areas of reduced myocardial perfusion indicative of CAD. PET, while less commonly available, provides higher accuracy and better quantification of myocardial blood flow, and may be particularly useful in certain complex cases.
- High Pre-test Probability (>85%): In these patients, direct invasive strategies such as coronary angiography are often considered appropriate due to the high likelihood of significant coronary artery disease. However, PET can be used in specific scenarios to assess myocardial viability, especially when considering revascularization options.

Coronary Computed Tomography Angiography (CTA) is the preferred test in patients with a lower range of clinical likelihood of CAD, no previous diagnosis of CAD, and characteristics associated with a high likelihood of good image quality. It detects subclinical coronary atherosclerosis but can also accurately rule out both anatomically and functionally significant CAD. It has higher accuracy values when low clinical likelihood populations are subjected to examination [3]. Trials evaluating outcomes after coronary CTA to date have mostly included patients with a low clinical likelihood [4, 5].

The non-invasive functional tests for ischemia typically have better rule-in power. In outcome trials, functional imaging tests have been associated with fewer referrals for downstream coronary angiography compared with a strategy relying on anatomical imaging [6-8].

The clinical significance of high-risk ischemic patterns

Before revascularization decisions can be made, functional evaluation of ischemia (either non-invasive or invasive) is required in most patients. Therefore, functional non-invasive testing may be preferred in patients at the higher end of the range of clinical likelihood if revascularization is likely or if the patient has previously been diagnosed with CAD.

When severe myocardial ischemia, indicative of substantial coronary artery obstruction, is identified, it represents a key determinant in the decision-making process for proceeding with interventional procedures. Patients displaying severe ischemia are often recommended for coronary angiography, which can lead to interventions such as percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG). These procedures aim to restore adequate blood flow to the ischemic areas, thereby improving symptoms, cardiac function, and overall prognosis [9, 10].

The ISCHEMIA (International Study of Comparative Health Effectiveness with Medical and Invasive Approaches) trial provided significant insights into the impact of the extent of myocardial ischemia on therapeutic decision-making in patients with stable CAD.

This trial explored the outcomes of patients with moderate to severe ischemia who were treated either with conservative medical therapy alone or with an initial invasive strategy involving angiography and possible revascularization [11].

The trial demonstrated that the initial invasive strategy did not significantly reduce the risk of major cardiovascular events compared to medical therapy alone in the overall cohort. However, subgroup analyses suggested that patients with more extensive ischemia might benefit more from revascularization in terms of symptom relief and quality of life improvements [11].

These findings emphasize the importance of personalized treatment strategies based on the extent of ischemia. While the results challenge the necessity of routine invasive procedures for all patients with moderate to severe ischemia, they highlight the need for a tailored approach, considering the individual patient's ischemic burden and symptomatic status.

Clinicians are required to carefully assess the extent of myocardial ischemia using non-invasive imaging techniques in stable CAD patients [1].

The ESC Guidelines summarize the definitions of high event risk for the different test modalities in patients with established chronic coronary syndromes [1, 12-14]:

- Exercise ECG: cardiovascular mortality >3% per year according to Duke Treadmill.
- Score SPECT or PET perfusion imaging: area of ischemia ≥10% of the left ventricle myocardium.
- Stress echocardiography: ≥3 of 16 segments with stress-induced hypokinesia or akinesia.
- CMR: ≥2 of 16 segments with stress perfusion defects or ≥ 3 dobutamine-induced dysfunctional segments.

The role of nuclear medicine in patients with suspected CAD

Nuclear medicine is a critical component in the field of cardiology, offering diagnostic and prognostic insights that are essential for the effective management of heart diseases. This branch of medicine utilizes radioactive substances, known as radiotracers, to create images of the heart and study its function and structure in detail.

Both SPECT and PET play significant roles in assessing the likelihood of ischemic heart disease based on pre-test probabilities [1]. Guidelines outline specific scenarios in which SPECT and PET are particularly valuable, emphasizing their utility in refining diagnostic accuracy and guiding clinical decision-making [1].

Myocardial perfusion imaging with SPECT has been generally regarded as the reference standard for the evaluation of myocardial perfusion [1]. SPECT imaging is a robust tool for diagnosing CAD by evaluating myocardial perfusion deficits during stress testing. It is particularly useful for assessing the severity and extent of ischemia, helping to guide decisions about the necessity for angiography or revascularization [1].

Otherwise, PET offers several advantages over SPECT, including higher spatial resolution, the ability to quantitatively assess myocardial blood flow, and reduced radiation exposure to the patient [1]. PET is highly effective in evaluating myocardial viability and differentiating between scarred and hibernating myocardium, which is crucial for planning revascularization in patients with severe ischemia or complex coronary anatomy [1].

Both SPECT and PET should be integrated into the clinical pathway according to the patient's individual risk profile, symptoms, and initial test results. The guidelines recommend using these imaging modalities to refine risk stratification, particularly in intermediate-risk

patients, and to guide further invasive diagnostic or therapeutic procedures based on imaging findings.

A patient-centric approach, using the best available diagnostic tools to inform treatment strategies, thereby optimizing care for patients with suspected or confirmed CAD, is advocated to apply the best cost-effectiveness approach to an increasing disease.

Acknowledgements

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BPIFB4 protein and monocyte phenotyping: a preclinical asset for marking the frailty condition

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Frailty; longevity; monocytes; biomarker

ABSTRACT

Frailty is a state of increased vulnerability to stressors arising from the systemic decline in physiological reserve mechanisms with aging. Advanced age impacts on frequency and phenotype of immune cells such as monocytes and macrophages. BPIFB4, a host defense protein with immunomodulatory activity, is protective in healthy long-living individuals in whom monocytes and macrophages have a favorable redistribution and phenotype. Although we reported an inverse correlation of the homozygous LAV-BPIFB4 haplotype with frailty in elderly subjects, the role of the circulating BPIFB4 levels as a frailty biomarker has not yet been characterized. In this study we investigated the correlation between BPIFB4 levels and both the frailty assessment/health status and monocytic profile in frail subjects.

Participants (40 frail individuals and 20 age-matched healthy volunteers) were subjected to standardized questionnaires to assess frailty risk, routine clinical examinations and blood tests; monocytes were analyzed by flow cytometry.

Overall, 70% of the frailty cohort had mild frailty, 25.5% had moderate frailty, and 5% had severe frailty. Compared to healthy controls, frail subjects showed lower levels of circulating BPIFB4 that inversely correlated with the relative risk index for hypertension and cardiovascular disease. The total circulating monocyte frequency is reduced in frail subjects compared to healthy controls. CD14++CD16– classical monocytes and CD14+CD16++ non-classical monocytes were significantly increased in frail people compared to healthy controls, whereas intermediate CD14++CD16+ monocytes were reduced. The M2/M1 monocytic balance was also altered in frailty condition. No relationship between BPIFB4 plasma levels and monocytes' subsets was found.

Our findings highlight that BPIFB4 protein has a potential prognostic value for marking the frailty condition.

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Introduction

People worldwide can expect to live longer. By 2030, about 20% world's population will be aged 60 years or over, which is why frailty is expected to reach epidemic proportions in the coming decades. Frailty represents an age-related dysregulation of the physiological functions and reserve mechanisms associated with adverse health outcomes. A state of vulnerability to stressors and dysfunctional homeostasis persists in frail subjects [1]. Frailty is recognized by clinicians, but its definition requires a complex systemic approach that takes into account biological and psychosocial correlates, and single symptoms are not sufficient to highlight it [2, 3]. Precisely because of its syndromic nature, the research area lacks an operational assessment tool for frailty that meets international consensus [4-6]. Among the various frailty assessment tools, we particularly highlight Fried et al. [7] frailty phenotype and Rockwood's [8] cumulative deficit model, which have achieved an international reputation. The frailty phenotype ranges from not-frail to pre-fail and frail, [7], and the different frail states are gradually strongly associated with a higher risk of

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developing adverse geriatric outcomes. In agreement with Wleklik et al., frailty develops in 25% to 62% of patients with cardiovascular diseases and, meanwhile, the presence of CVDs implies the increased risk of developing frailty in older people [9]. Furthermore, multimorbidities (such as hypertension, diabetes, and COPD) which are common in old and frailty, pose a detrimental predictor of health outcomes in older patients [10]. In the aging context, long-living individuals constitute a model of exceptional healthy aging, considering their ability in overcoming and coping better with age-related diseases and frailty, despite their biological age. Previous work from our group identified a longevity-associated variant (LAV) of BPIFB4 associated in homozygosity with exceptional longevity in different independent populations [11, 12]. The bactericidal/permeability-increasing fold-containing family-B-member-4 (BPIFB4) is a secreted protein highly abundant in respiratory secretions, in the upper airways and proximal trachea. Besides the longevity-associated variant (LAV), which is constituted by the minor allele of rs2070325 that is part of a four SNPs haplotype, the gene BPIFB4 presents other two isoforms: the wild type (WT)-BPIFB4, which is constituted by major alleles of the four SNPs, and the rare-variant (RV)-BPIFB4, found to be a biomarker of vascular dysfunction and hypertension [11, 13]. Compared to the other two isoforms, LAV-BPIFB4 gene transfer was found to exert advantages by reducing atherosclerosis progression and inflammation in ApoE$^{-/-}$ mice [14], by contrasting immunosenescence and aorta senescence in a murine model of advanced age, [15], by restoring the heart function in a model of diabetic cardiopathy [16]. Furthermore, the serum of long-living individuals is enriched in BPIFB4 compared to controls and frail people, thus classifying their health status [17]. Moreover, Malavolta et al identified LAV-BPIFB4 haplotype was significantly under-represented in frail subjects and the LAV-BPIFB4 gene therapy in old mice clinically attenuated the progression of frailty [18]. LAV-BPIFB4 also showed an interesting involvement with regard to the immune compartment. Indeed, the longevity-associated variant of BPIFB4 showed the ability in driving both dendritic cells toward a regulatory phenotype [19] and the macrophage-skewing toward a pro-resolving M2 phenotype in atherosclerotic subjects [14]. Considering the age-related changes in innate immune cells and that circulating BPIFB4 levels were found to associate with the abundance of pro-resolving monocytes and macrophages in long-living individuals [20], here we evaluate the profile of monocytes and macrophages in recruited frail subjects compared to healthy volunteers and the potential correlation with BPIFB4 circulating levels. Indeed, more efforts are needed to find optimal biomarkers associated with frailty capable of being valuable for the early diagnosis or prognosis of frailty in older people. From a translational point of view, the main purpose of this work was to evaluate the possible usefulness of BPIFB4 as a prognostic tool for marking frailty.

Materials and methods

Study design and sample characteristics

The study is a single-center, cross-sectional survey conducted between January 2016 and January 2017 among a group of older patients recruited from a random sample stratified by age and gender, at the Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana", University of Salerno and the University Hospital "San Giovanni di Dio e Ruggi d'Aragona", Salerno Italy. The primary objective was to assess, through a validated questionnaire and clinical examinations, the health status and frailty index of the young old and the old/great old, respectively. The secondary objective was to understand, through blood tests, whether the immunophenotype and genotypic characterization had a possible correlation with frailty. It is important to use screening and assessment tools to investigate the different dimensions of health and identify the frailty condition earlier to help patients recover function and prevent adverse outcomes.

The study was performed on a group of 67 individuals, n=47 frail patients and n=20 aged-matched healthy volunteers free from risk factors for, and clinical evidence of clinical signs and symptoms of relevant communicable disease agents and chronic diseases, and treatments related to medical conditions.

For each patient, venous blood (10 mL) was withdrawn for analyses and detailed anamnesis was collected. All participants signed an informed consent for the management of personal anamnestic data and blood samples. The study was approved by the Campania Sud ethical committee and conducted in accordance with the ethical principles deriving from the Declaration of Helsinki (N.78 _r.p.s.o. del 04/07/2018." *Studio per la valutazione della correlazione tra le isoforme del gene BPIFB4 e il rischio di fragilità umana*").

Of the forty-seven frail patients recruited, 40 were selected and completed the study, as the eligibility criterion was that the patients' phenotype fell within the threshold value of frailty [7], that the patients were aged 65 to 90 years or older and that there were no obvious disabilities; seven fell within the exclusion criteria as they did not have the above characteristics and belonged to the robust subjects [1]. The patients met the criteria outlined in international clinical practice guidelines for the identification and management of frailty in older adults [21].

Data collection for baseline evaluation

Standardized questionnaires ascertained self-rated health status, health habits, weight loss, and self-reported medical diagnoses of cardiovascular events (hypertension, angina pectoris, chronic heart failure, stroke), diabetes, chronic pulmonary disease, and cancer.

The multidimensional procedure "Comprehensive Geriatric Assessment" was used to assess the functional ability [22, 23], physical, cognitive, and mental health [24, 25], and socio-environmental status [26, 27] of older patients. Functional status was ascertained by asking old patients whether they had difficulty performing 12 tasks of daily living, tasks included in instrumental activities of daily living (IADLs) and activities of daily living (ADLs) [28]. Physical function was assessed with several questions from the Physical Activity Scale for Elderly (PASE) [29], which includes standardized performance-based measures of physical function, such as time (seconds) taken to walk 4 meters [25] and grip strength (kilograms) of the dominant hand (2 measures on mean), using a Smedley handheld dynamometer. Cognitive and mental health was assessed with the Mini-Mental State Examination (MMSE) [30] and the Geriatric Depression Scale (GDS) [24]. The Social Support Assessment (SSA) was used to assess whether older patients had social relationships and, if so, whether the level of support was high, fair or low [27]. Through standardized clinical examinations, such as electrocardiogram, echocardiography, and pressure report, and subsequent evaluation of the data by physicians, cardiovascular diseases (hypertension, angina pectoris, chronic heart failure, stroke) were validated [31].

Further examinations ascertained: body weight (kg) and height (cm) to calculate body mass index (BMI); blood test to determine fasting glucose level; and M1/M2 immunophenotypic analysis and genotype characterization.

Rockwood frailty index data

Rockwood's Frailty Index was calculated using information collected during various routine health assessments of older adults,

specifically 38 variables were considered to have an accurate index [32, 33]. Issues related to functional difficulties, such as difficulty in washing, dressing, sitting or getting up from a chair, walking, eating, taking care of the house, using the toilet, climbing or descending stairs, grocery shopping, household chores, preparing meals, taking medication, managing money, staying in bed at least half the day due the health, and reducing habitual activity, were coded as binary variables, using the convention that "0" indicates no deficit and "1" indicates the presence of a deficit. For the self-rated health question "How do you rate your health?" a six-point Likert scale was used for responses, where the endpoints are labeled 0 = excellent, 0.25 =very good, 0.5 = good, 0.75 = poor, and 1 = poor. For the question "Has your health changed in the last year", the response includes 0 = better/same, 1 = worse.

Standardized measures, to define physical health, including time taken to walk 4 meters, the cutoff of which was coded as a binary variable, where 1 ≥10 frailty index criterion and 0 ≤10 non-frailty index criterion; and grip strength, which was stratified into quartiles based on gender and body mass index (BMI) [7] and then recoded into binary as follows:

1 male = presence of grip strength if BMI ≤24 and kg ≤29; BMI 24.1- 26 and kg ≤30; BMI 26.1- 28 and kg ≤30; BMI >28 and kg ≤32.

0 male = absence of grip strength if BMI ≤24 and kg >29; BMI 24.1-26 and kg >30; BMI 26.1-28 and kg >30; BMI >28 and kg >32.

1 female = presence of grip strength if BMI ≤23 and kg ≤17; BMI 23.1- 26 and kg ≤17.3; BMI 26.1 -29 and kg ≤18; BMI >29 and kg ≤21.

0 female = absence of grip strength if BMI ≤23 and kg >17; BMI 23.1-26 and kg >17; BMI 26.1- 29 and kg >18; BMI >29 and kg >21.

Variables related to cognitive health (GDS), such as "feeling that everything is an effort", "feeling depressed" and "feeling happy", were coded through a three-point Likert scale, 0 = rarely, 0.5 = sometimes, 1 = most of the time. Regarding the Mini-Mental State Examination (continuous variable), recoding was done according to the severity of impairment [31], assigning 1 for scores <10 defined as "severe dementia", 0.75 for scores ≥10 and ≤17 classified as "moderate dementia", 0.5 for scores ≥ 18 and ≤20 defined as "mild dementia", 0.25 for scores >20 and <24 "mild cognitive impairment" (MCI), and 0 for scores ≥24 "no cognitive impairment" [33].

The comorbidities were assessed both as a cumulative total and as a single disease, such as hypertension, diabetes, chronic obstructive pulmonary disease (COPD), chronic heart failure, angina pectoris, stroke, and cancer, labeled with a three-point Likert scale with endpoints such as 0 = no disease, 0.5 = suspected presence, 1 = presence of disease.

By dividing weight (kg) by height squared (m^2), body mass index (BMI) was calculated in old patients. The BMI (variable continuous) was recoded, according to the criteria established by the WHO, considering 0 = normal weight, 0.5 = overweight, and 1 = obese. The mini-nutritional assessment (MNA) was coded into three-point Likert scales labeled as 0 if the score is between 24 and 30 and shows "normal nutritional status", 0.5 if the score is between 17 and 23.5 "risk of malnutrition", and 1 if the score is < 17 "malnutrition" [17].

Social Support Assessment (SSA) has been coded to 0 "low social support" if the range is 0 to 2.9; 1 "moderate support" if the range is 3 to 5.9; and 2 "high support" if the range is 6 to 10 [27].

The frailty index was calculated based on the score of the deficits present in the patients in relation to the total number of deficits considered. Based on severity, the frailty index was divided into "mild" if the score was between 0 to 13.9, "moderate" if it was between 14 to 24.9, and "severe" if it was between 25 to 38.

Flow cytometry and immune phenotypical analysis

Peripheral blood mononuclear cells (PBMC) were extracted from whole blood by density gradient (Ficoll). After separation, PBMC were collected and washed for the subsequent experiments.

Conjugated monoclonal antibodies against CD14, CD16, CD86, and CD163 were purchased from BD Biosciences. After 20 minutes of incubation at room temperature in the dark, cells were washed with staining buffer and resuspended for the FACS analysis. For each test, cells were analyzed using a FACS Verse Flow Cytometer (BD Biosciences).

ELISA assay

Plasma levels of BPIFB4 were measured using an ELISA Kit (Cusabio CSB-YP003694HU) following the manufacturer's protocol. Concentration values were subjected to statistical analysis by using GraphPad Prism 6.0 software for Windows (GraphPad software).

Genotyping

Genetic analysis for the SNP rs2070235 (p.Ile229Val) on BPIFB4 was assessed in all subjects. From all samples collected, leucocytes were used to extract their genomic DNA (DNeasy kit, Qiagenâ). Then, the DNA was quantified to normalize concentrations run on quantitative polymerase chain reaction (PCR)-Taqman-based method.

Statistical analysis

Descriptive statistics were used to summarize patients' characteristics considering perception of current and last-year health status, diseases at baseline, and frailty indexes; responses to all items were shown with absolute and relative frequency values for categorical variables and mean and standard deviation for continuous variables. Multivariate analysis plots were used to show changes in values of the multidimensional comprehensive geriatric assessment (for better understanding, CGA data were recoded into 5-point Likert with endpoints labeled as -2 worst health status and 2 best health status). Poisson regression analysis was performed to calculate significant predictors of BPIFB4 protein and Rockwood frailty index on the measured variable. The incidence ratios (IRRs) and their 95% confidence intervals (CIs) were used in the Poisson regression models to measure the independent associations between the different variables and the outcomes of interest. Pairwise correlation analysis was performed between BPIFB4 protein and patrolling. For all analyses, values of 0.05 or less were considered statistically significant. Data analyses were conducted using STATA software (Release 16.1, StataCorp LLG, College Station, TX, USA, 2019).

Results

Demographic characteristics, perceived health status, and frailty indexes

Forty people, 35% female and 65% male, with a mean age of 73.5±5.4 (range 65-86 years), with different health conditions and frailty were evaluated (**Table 1**). Overall, 70% of the cohort had mild frailty, 25.5% had moderate frailty, and 5% had severe frailty. 77.5% of patients rated perceived health as fair to good, while perceived health in the last year was rated worse for 65% of the old.

Among chronic diseases, the highest rates are evident for hypertension, cardiovascular heart failure, and diabetes (**Table 1**).

Comprehensive geriatric assessment of patients

Figure 1 shows the results of the comprehensive geriatric assessment. Physical health, assessed through standardized measures of

Table 1 | Baseline patient and disease characteristics of the 40 analyzed patients and 20 healthy controls enrolled in the study.

Patients characteristics (Tot sample = 40)	N	%	Healthy controls (Tot sample = 20)	%
Gender				
Female	14	35	7	35
Male	26	65	13	65
Age	73.5 ± 5.4 (65-86)		70 ± 4.9 (65-75)	
Perception of current health status				
Poor	2	5	–	–
Fair	11	27.5	–	–
Good	20	50	12	60
Very good	4	10	7	30
Excellent	3	7.5	1	10
Perceived health status in the last year				
Worse	26	65	N/A	N/A
Better	14	35	N/A	N/A
Prevalent Disease at Baseline				
Hypertension	32	82.5		
Diabetes	15	38.5		
COPD	14	35.9	N/A	N/A
CHF	28	71.8		
Angina pectoris	10	25.6		
Stroke	4	10.2		
Cancer	1	2.6		
Rockwood frailty index (RFI)				
Mild	28	70		
Moderate	10	25.5	N/A	N/A
Severe	2	5		

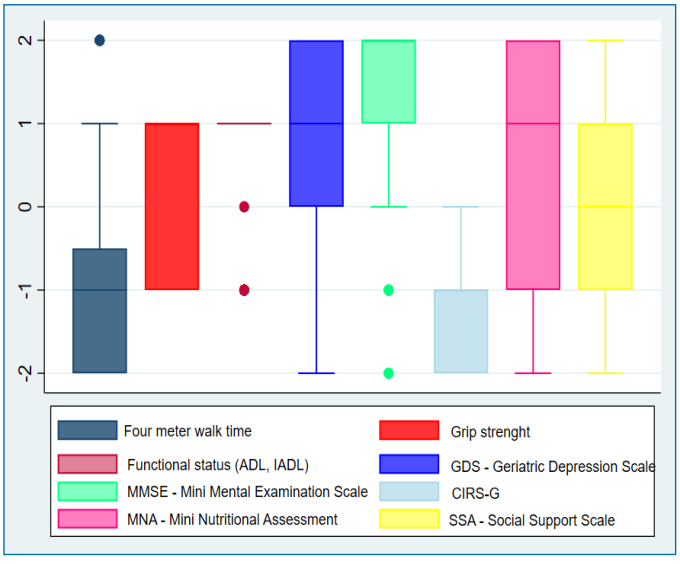

Figure 1 | Comprehensive geriatric assessment of patients. *Note: CIRS-G Cumulative Illness Rating Scale in Geriatrics; ADL Activity of Daily Living; IADL Instrument Activity Daily Living.*

some performance, including time taken to walk 4 meters, showed that 75% of the patients took longer than the established standard; while for grip strength, 2/4 of the older people presented grip ability. Regarding functional status, delineated through activities of daily living (ADL, IADL), it was inferred that 1/4 of the cohort had functional deficits.

Cognitive and mental health was good, 2/3 of the old did not suffer from depression, and only 17.5% had moderate or mild dementia. As for comorbidities, they were particularly evident in each patient. Regarding the screening of nutritional status, 1/3 of the patients had a risk of malnutrition. Finally, the social support need assessment showed that patients were equally divided between those who had high support and those who had low support.

Analysis of BPIFB4 blood levels in frail patients

The bactericidal/permeability-increasing fold containing family B member 4 (BPIFB4), characterized as both a longevity-associated and a host defense protein with a proven immunomodulatory activity (19, 20, 34), displayed prognostic relevance and inversely correlated with disease severity in COVID-19 and atherosclerosis. Furthermore, circulating levels of BPIFB4 are increased in healthy Long Living Individuals LLIs as compared to old controls [20] as a putative biomarker of life-long expectancy.

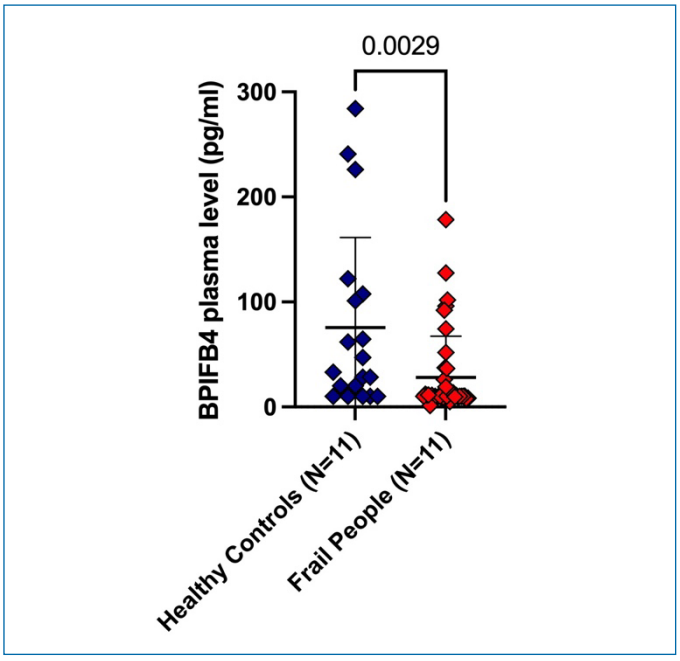

Figure 2 | ELISA quantification of BPIFB4 plasma levels in Healthy control and Frail People (non parametric Mann-Whitney U test).

We examined the plasma BPIFB4 levels in N=40 frail people and N=20 healthy controls, for comparison (**Figure 2**). Notably, BPIFB4 values were significantly lower in frail individuals as compared with old controls pointing to BPIFB4 as a bona fide biomarker inversely related to frailty condition.

Significant predictors of BPIFB4 protein, genotype, and Rockwood frailty index

The Poisson regression model constructed to study the relationship between BPIFB4 protein and patients' comorbidities showed a significant protective effect for hypertension (IRR = 0.32; 95% CI 0.12-0.844; p = 0.02) and cardiovascular disease (IRR = 0.50; 95% CI 0.26-0.97; p = 0.04), while there were no significant relationships with diabetes, COPD and stroke (Model 1 **Table 2**). Furthermore, Poisson regression results revealed that statistically significant predictors of RFI were homo/hetero genotype (IRR = 0.78; 95% CI 0.64-0.95; p = 0.01) (Model 2 **Table 2**).

Characterization of monocytic dynamics in frail elderly

The frequency and phenotype of different immune cell populations are severely affected by the advanced age and its related comorbidities. Our data demonstrate that total circulating monocyte frequency is significantly reduced in N=11 immunophenotyped frail subjects as compared with healthy controls (**Figure 3**).

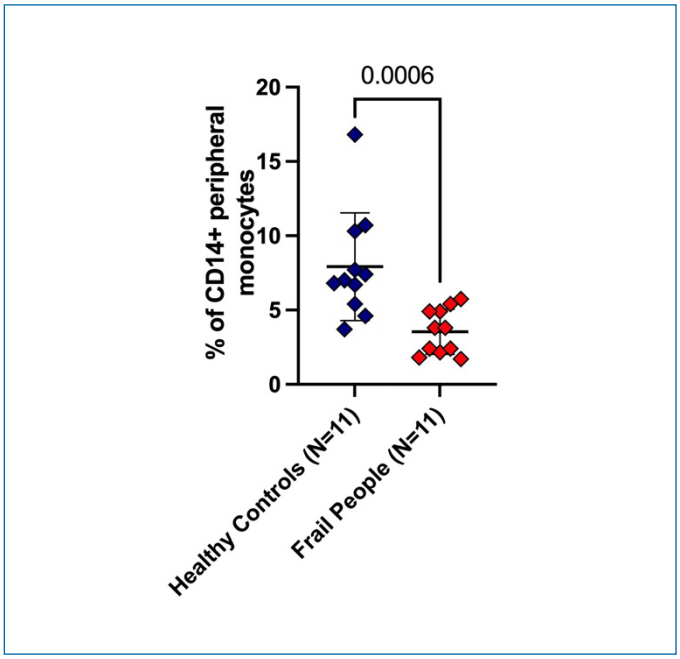

Figure 3 | Frequency of monocytes in Healthy Controls (N=11) and Frail People (N=11) expressed by percentage of total CD14+ positive cells in cytofluorimetric analysis. (non parametric Mann-Whitney U test).

Table 2 | Correlation analysis between BPIFB4 protein and patients' comorbidities.

Model 1. BPIFB4 (sample size = 39) Log likelihood = -50.03, x^2 = 15.42 (6 df), p = 0.017	**IRR**	**95% CI**	**p**
Hypertension	0.32	0.12 - 0.84	0.02
Diabetes	1.03	0.55 - 1.94	0.91
COPD	0.79	0.41 - 1.53	0.49
CHF	0.50	0.26 - 0.97	0.04
Angina pectoris	0.87	0.36 - 2.12	0.77
Stroke	1.11	0.23 - 17.22	0.89
Model 2. RFI (sample size = 39) Log likelihood = -116.11, x^2 = 6.28 (2 df), p = 0.043			
BPIFB4	0.99	0.99 - 1.00	0.50
Genotype			
WT	1	1	1
homo/hetero	0.78	0.64 - 0.95	0.01

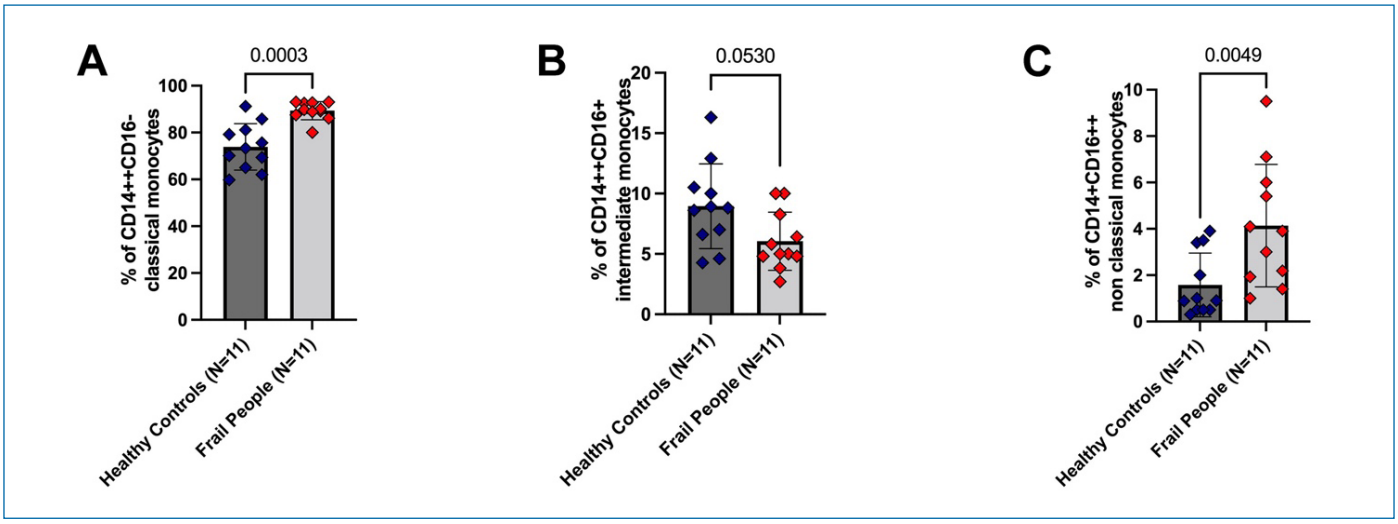

Figure 4 | FACS analysis of monocyte subpopulations in the healthy control group (N=11) and frail people group (N=11). In (A) is expressed the % of CD14++CD16- Classical monocytes, which appears to be increased in Frail People versus Healthy controls). In (B) it is expressed the % of CD14++CD16+ Intermediate monocytes that result decreased in Frail People compared to Healthy controls while in (C) it can be appreciated the difference in % of CD14+CD16++ Non-Classical monocytes subgroup that is increased in Frail People (non parametric Mann-Whitney U test).

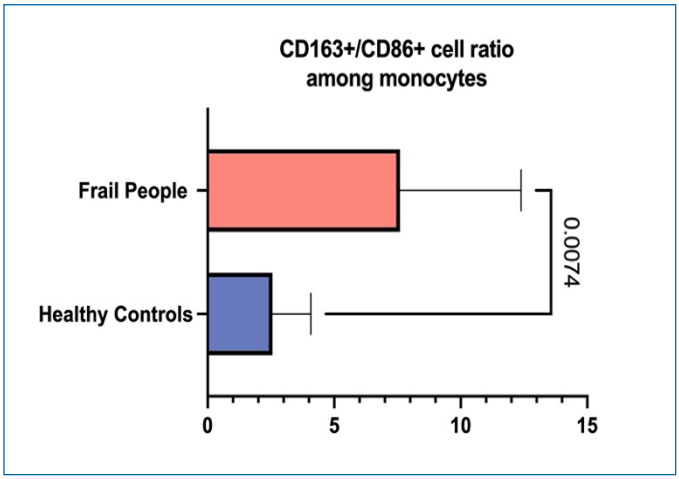

Figure 5 | CD163+/CD86+ ratio in CD14+ cells in Frail People (N=11) and Healthy Controls (N=11) (non parametric Mann-Whitney U test). In Frail People there is a higher ratio, meaning that CD14+CD163+ M2 monocytes are more abundant compared to CD14+CD86+ M1 monocytes.

Looking for differences in subsets of monocytes, CD14++CD16– classical monocytes and non-classical CD14+CD16++ monocytes were significantly increased in frail people compared to old controls, whereas intermediate CD14++CD16+ monocytes were reduced (**Figure 4**).

Moreover, when we profiled total CD14+ monocytes according to their surface levels of

CD86, a classical M1 pro-inflammatory marker, and CD163, a canonical M2 pro-resolutive marker, we described an altered balance of M2/M1 in frailty conditions compared to old volunteers (**Figure 5**).

As levels of CD163 are strongly regulated by mediators in the inflammatory response [35], its enhanced expression on monocytes from frail elderly may be a potential biomarker reflecting efforts by the immune system to resolve immune activation and inflammation (typically referred to as *inflammaging*).

Correlation analysis between BPIFB4 and circulating monocyte subsets.

As in LLIs BPIFB4 levels are associated with a favorable redistribution of monocyte compartment and macrophage polarization *in vitro,* we asked if the reduced BPIFB4 levels in frail people may dictate or contribute to the altered monocyte frequency and phenotype (**Table 3**).

Table 3 | Correlation analysis between BPIFB4 protein and patients' monocyte pool.

	BPIFB4	Non-classical monocytes	Intermediate monocytes	Classical monocytes	CD14 monocytes frequency
BPIFB4	1.000				
Non-classical monocytes	0.159	1.000			
Intermediate monocytes	0.021	0.179	1.000		
Classical monocytes	-0.100	-0.742*	-0.784*	1.000	
CD14 monocytes frequency	0.209	0.199	-0.184	0.019	1.000

Note: * significant correlation with p value ≤0.05.

Correlation analysis between the BPIFB4 protein and all the monocytes' subsets showed no relationship. Statistical significance was shown only between classical monocytes and non-classical monocytes ($p = 0.008$) and between classical monocytes and intermediate monocytes ($p = 0.004$).

Discussion

The main objectives of this study were to assess the health status and frailty index of a group of young old and old/great old, respectively, and to present associative clinical evidence between frailty and both frailty-specific protein biomarkers and immunophenotypically peculiar assets. Frailty is considered a complex and multidimensional syndrome influenced by both clinical features and social and environmental determinants of health. Frail people have a multisystemic reduction in normal physiological functions leading to increased vulnerability to stressful events and a reduced ability to restore homeostasis [4, 36]. The old population is normally characterized by a progressive loss of physiological reserves, but in frailty, this mechanism is even more evident [37]. Frailty is known to be associated with increased adverse sequelae [38, 39], depression [40], reduced self-sufficiency [41], fractures [42], cognitive impairment [43], hospitalization [44, 45], the need for long-term care interventions [41], reduced levels of quality of life [46, 47], and premature death [48, 49]. Therefore, in our study, using Rockwood and Mitnitski's model [33], a frailty index was constructed that provides a holistic view of different dimensions of health to identify this condition early, helping patients to prevent associated adverse outcomes. The older one gets the greater the risk of developing chronic diseases and multimorbidity (presence of two or more chronic diseases) situations [50, 51]. Accordingly, our results showed that comorbidities were particularly evident in each patient. Although the presence of multiple chronic conditions is associated with the development of frailty [52], frailty is not necessarily the result of chronic disease. On the other hand, it is also true that intensive or overtreatment of chronic diseases can increase adverse health outcomes in frail people [52, 53] as clarified by Elliot et al. [54] who claimed that frailty can hinder adherence to both pharmacological and rehabilitative therapies. Several studies have shown that a cardinal manifestation of frailty is loss of physical function [55]. Poor physical function, muscle atrophy, and dyspnea are shared conditions between the phenotypic model of frailty and sarcopenia. The approach to the diagnosis of sarcopenia involves the search for symptoms such as falls, weakness, slowness, self-reported muscle atrophy, or difficulty performing activities of daily living [56, 57]. According to the European Working Group on Sarcopenia in Older People (EWGSOP) (56], the approach to the diagnosis of sarcopenia should be stepwise and begins with the measurement of muscle strength, usually grip strength, following which sarcopenia may be suspected. In our research, the results of standardized measures of some performances, including time taken to walk 4 meters, grip strength, and activities of daily living (ADL, IADL), showed that half of the cohort analyzed had suspected sarcopenia. In agreement with Coelho-Junior et al. [58], the risk is that a physically inactive lifestyle may lead to the progression of both conditions. In support, Landi et al. [59] evaluating this scenario, proposed sarcopenia as the biological substrate of frailty, while Marzetti et al. [60] combined the two conditions into a new clinical entity called physical frailty-and-sarcopenia. On the other hand, the pathophysiology of frailty and sarcopenia may have a multifactorial etiology involving many of the biological features of aging (e.g., genomic and epigenetic instability, loss of proteostasis, mitochondrial shortening, telomere shortening, stem cell depletion, cellular senescence) [61, 62].

In agreement with Solfrizzi et al. [63] who studied cognitive and mental components, they deduced a correlation between physical frailty and cognitive impairment, defined as cognitive frailty. The cognitive and mental health of the patients in our study was good, most of the old people examined did not suffer from depression, and only a small percentage (1/4) had moderate or mild dementia. It is important to remember, however, that cognitive frailty, like physical frailty, can be delayed at least in the early stages and that its presence can lead to an increased risk of events that negatively impact health [64], such as worsening quality of life [65], increased hospitalizations and mortality [66].

It is well known that health is a reflection of several factors in addition to biomedical factors, such as social [67] and environmental factors [68, 69]. Social determinants of health, such as work, social networks, eating habits, and internal and external living environment, can indeed decrease an individual's intrinsic and extrinsic abilities, making him or her frail. The results of our study showed that patients were equally divided between those with high support and those with low support. In agreement with Aliberti et al. [26], the old person needs the support of family and third parties, as well as cultural activities and recognition can have a significant impact in terms of personal well-being. Azzopardi et al. [67] in the context of frailty, point out that social aspects and especially environmental and personal (e.g., relational) factors of an individual are not sufficiently considered by social and health professionals.

Collectively, the results of our study showed that two-thirds of patients had mild frailty, so it was possible to intervene to help patients regain function. As changes in the proteome and the degree of peripheral immune response are also related to the progression into frailty [70], we propose protein biomarkers and immune traits related to the frailty condition and its progression. Indeed, the diagnosis of frailty is usually clinical and based on selected criteria, which are sometimes inconsistent. Therefore, there is an urgent need to identify and validate novel biomarkers.

While most popular circulating markers are those related to the inflammatory response (eg, C-reactive protein [CRP], IL6, and tumor necrosis factor-alpha [TNFα]) or oxidative stress and/or hormones (insulin-like growth factor-1 [IGF1], testosterone) [71] here for the first time the peculiar asset of monocytes and BPIFB4 circulating factor may constitute new disease biomarkers and therapeutic opportunities in the complexity of the frailty condition. BPIFB4 has already been shown to serve as a biomarker of healthy aging [14 , 20, 72] and display prognostic significance in vascular pathology and COVID-19 [73] mainly influencing mono-macrophage skewing. Here BPIFB4 plasma levels are inversely correlated with frailty condition even though no significant correlations were found between BPIFB4 and different monocyte subsets characterizing frail elderly. Noteworthy, we corroborated a decline in monocyte frequency in frail people compared to the non-frail group. The peripheral reduction of monocytes may suggest their robust recruitment in the damaged tissue as also suggested by the higher levels of monocyte chemoattractant protein-1 (MCP-1) characterizing frail vs non frail-group [74]. The functional changes in peripheral monocyte response deserve much attention as they reveal an expected inflammatory arm (CD14++CD16- Classical monocytes *high*) but counterbalanced by a reparative polarized activity (CD14+CD16++ Non-Classical monocytes *high* and CD14+CD163+ monocytes *high*) of monocytes. Indeed, as levels of CD163 are strongly regulated by mediators in the inflammatory response [35], its enhanced expression on monocytes from frail elderly may be a potential biomarker reflecting efforts by the immune system to resolve immune activation and inflammation (typically referred to as *inflammaging*). On

the other hand, the prevalence of CD14+CD16++ Non-Classical monocytes and CD14+CD163+ monocytes failing to be activated may reflect an exhausted state of the frail vs non-frail group. This scenario also emerged from the transcriptome on the single-cell level in human-aged immune cells from frail (n = 5; age, 88.0 ± 5.8 years) individuals compared to healthy old individuals (n = 6; age, 85.8 ± 11.1 years) [70].

Even though not investigated in our report, probably a different monocyte balance in the dynamic course of the frailty (pre-frail/frail) may be useful to dissect the pathophysiological process and its clinical progression. The lack of association with BPIFB4 levels can be in part explained by the sample size, which is an obvious limitation of our study, or probably because high BPIFB4 levels correlate with pro-resolutive M2 response only when resulted protective and useful to blunt inflammatory tone [14, 15, 75, 76], while in this context the CD163+/CD86+ balanced ratio contributed to the impaired nature of frail monocytes. Likewise, monocyte variations in some frail individuals can reflect a plethora of comorbidities-associated states for some of which BPIFB4 levels do not guarantee a proper degree of protection. Indeed, here BPIFB4 levels resulted protective for hypertension and cardiovascular disease, while there were no significant relationships with diabetes, COPD, and stroke (Model 1 **Table 2**). This is consistent with the cardiovascular benefits of carrying the LAV isoform of the BPIFB4 gene associated with healthy aging and a high degree of protection from hypertension, ischemia, and atherosclerosis. Furthermore, the association of the LAV haplotype with lower frailty in elderly subjects and the reduced frailty observed in mice treated with LAV-BPIFB4 gene therapy are in perfect agreement [18]. Here we confirmed a protective incidence relationship between frailty and homo/hetero BPIFB4 genotype (Model 2 **Table 2**). This genetic association may be strengthening the diagnosis of frailty which can take advantage of biomarkers, genetic and proteomic research, and incorporation of sociodemographic variables associated with frailty.

Conclusions

The epidemiological transition has led to a longer life expectancy with an increase in chronic diseases compared to acute diseases [77]. This may predispose to a progressive, whole-organism process of decompensated homeostasis with a substantial contribution from the impaired immune responses.

However, frailty is often reversible [78] in the early stages, before the onset of functional impairment. Therefore, early identification, through protein biomarkers and immunophenotypically at the pre-fragility or mild frailty stage, is important to help patients regain function and prevent adverse outcomes associated with the syndrome [79]. Our findings highlight that BPIFB4 protein has a potential prognostic value for marking the frailty condition deserving much attention in the near future.

Author contributions

E.C. designed and conducted the study, coordinated the research team, and wrote the manuscript. and S.M.A. performed statistical analysis and data interpretation and wrote the manuscript. F.M., V.L., C.B., A.C. and P.D.P., A.M. performed laboratory activities. M.C.C. and C.V. cared for the subjects of the study and evaluation of their health status and reviewed critically the paper. M.C. reviewed critically the paper. A.A.P. performed data interpretation and reviewed the manuscript. A.A.P. supervised the project in its entirety and provided financial support. All authors approved the final version to be published.

Competing interests

All authors declare no financial or competing interests that are directly relevant to the content of this manuscript.

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Declarations

Ethics approval and consent to participate.

The study was approved by the Campania Sud ethical committee and conducted in accordance with the ethical principles deriving from the Declaration of Helsinki (N.78 _r.p.s.o. del 04/07/2018." *Studio per la valutazione della correlazione tra le isoforme del gene BPIFB4 e il rischio di fragilità umana*"). All participants signed an informed consent for the management of personal anamnestic data and blood samples.

Consent for publication

Not applicable

Availability of data and materials

Data, materials, and protocols will be available on request by emails to the corresponding authors due to privacy/ethical restrictions.

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Effect of anti-PCSK9 drugs on the association of PCSK9 to LDL

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Keywords
Atherosclerotic cardiovascular disease; lipid-lowering therapy; monoclonal antibodies; small interfering RNA; LDL-cholesterol; PCSK9

ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein known to interact with the LDL receptor, thereby promoting its degradation and blunting the uptake of LDL from the circulation. In this context, anti-PCSK9 monoclonal antibodies (mAbs) and siRNAs have been approved for the treatment of hypercholesterolaemia. Previous studies have shown that a significant proportion of circulating PCSK9 is associated with LDL. The aim of our research is to investigate the effect of mAbs and siRNA on the association of PCSK9 protein with LDL. In this study, 10 statin-intolerant patients received treatment with anti-PCSK9 mAbs or siRNA, in addition to therapy with a low-dose statin and ezetimibe. Their plasma samples were analysed before and after 1, 3, and 6/9 months of treatment. The results showed that both the monoclonal antibodies and inclisiran reduced LDL-C levels by 50% to 60%. LDL-C levels decreased from 92±28 mg/dL to 44±26 mg/dL after siRNA treatment and reached 97±9, 27±10, 32±14, and 23±10 mg/dL after mAbs therapy. The circulating PCSK9 level decreased by 70% after the first siRNA injection, while it increased 10-fold after mAbs therapy. Regardless of treatment, the percentage of PCSK9 bound to LDL did not vary from baseline and remained constant during the treatment period. Whether this is of physiological relevance remains to be addressed.

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Background

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein that plays a crucial role in the regulation of plasma low-density lipoprotein cholesterol (LDL-C) levels [1]. It is primarily produced in the liver [2]. The PCSK9 protein binds to LDL receptors (LDLR) on the cell surface, leading to internalisation of the PCSK9-LDLR complex and targeting LDLR for lysosomal degradation, thus reducing the cell's ability to remove LDL-C from the bloodstream [3]. This leads to higher levels of LDL-C in the circulation and contributes to the development of atherosclerosis and cardiovascular disease [4, 5]. Lowering LDL-C levels is a key focus in the prevention and treatment of cardiovascular disease [6]. Lifestyle changes, including a healthy diet, regular exercise and avoiding tobacco use, can help to lower LDL-C levels. In addition, therapies such as statins are commonly prescribed to reduce LDL-C and lower the risk of cardiovascular events (7). The central role of PCSK9 in modulating LDL-C levels has driven the development of several approaches to inhibit this protein [8].

Evolocumab and alirocumab are fully humanised monoclonal antibodies (mAbs) that target circulating PCSK9 and have been investigated in several clinical trials, including the two outcome trials FOURIER and ODYSSEY OUTCOMES [9, 10]. Inclisiran is a small interfering RNA (siRNA) that specifically inhibits the hepatic synthesis of PCSK9 [11]. Both treatments lead to increased expression of LDLR in the liver, enhancing the removal of LDL-C from the blood [12, 13]. PCSK9 inhibitors are used as a therapeutic option to lower LDL-C levels in individuals with hypercholesterolaemia and a high risk of cardiovascular events [14]. Several studies are investigating the possible effects of PCSK9 inhibition beyond LDL-C levels [15]. In this study, we investigated the effect of mAbs and siRNA on the association of PCSK9 protein with LDL.

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Methods

Subjects and samples

For this study, we have selected 10 patients who were treated with a statin plus ezetimibe and experienced statin intolerance. Of these, 9 were classified as medium risk and 1 was classified as high risk for intolerance. Subsequently, the patients were divided into three groups, of which 5 subjects received a monoclonal antibody anti-PCSK9, 4 others received siRNA treatment, in addition to therapy with a low-dose statin and ezetimibe, and 1 patient replaced the statin with siRNA. Plasma samples were collected from each patient at baseline and after 1, 3 and 6/9 months of therapy. All blood samples were subjected to low-speed centrifugation (3000 rpm, 12 min) to obtain plasma to which the protease inhibitor (Halt™ Protease Inhibitor Cocktail, Thermo Fisher, Italy) was added. Each participant gave written informed consent for the study. This study was conducted in accordance with the Declaration of Helsinki.

Iodixanol density gradient ultracentrifugation

Lipoproteins were isolated from plasma using three layer-density of OptiPrep™ solvent as previously described [16].

Statistics

Statistical analyses were performed with GraphPad Prism 9.0. Data were analysed using the unpaired t-test and the one-way ANOVA test.

Results

The study cohort consisted of n=10 subjects, with 9 subjects being treated with alirocumab, evolocumab or inclisiran in addition to their existing therapy, while the remaining 1 subject was treated only with inclisiran. Plasma samples were collected before the therapies and at 1, 3, and 6/9 months (T1, T2 and T3, respectively) after the first injection. All samples were analysed for PCSK9 and lipoprotein distribution. Baseline levels of total cholesterol, LDL-C, HDL-C and TG of the subjects are reported in **Table 1**.

In patients receiving monoclonal antibody therapy, approximately 12% of the total PCSK9 was bound to LDL at baseline (T0). After one month of treatment with an anti-PCSK9 monoclonal antibody (T1), a 70% reduction in LDL-C levels (from 97±9 mg/dL to 27±10 mg/dL) was observed. Conversely, plasma PCSK9 levels increased 10-fold, from 562±156 ng/mL to 4,925±1400 ng/mL. Interestingly, the percentage of circulating PCSK9 bound to LDL remained unchanged throughout the therapy duration, despite the marked changes in LDL-C and PCSK9 levels. The data observed at T1 was confirmed at T2 and T3, where LDL-C levels were 27±10, 32±14 and 23±10 mg/dL, respectively; while PCSK9 concentration reached 4,925±1400, 5,360±755 and 5,843±920 ng/mL, respectively (**Figure 1**). The percentage of PCSK9 bound to LDL remained consistent with the level observed at T1, reaching values of 15% at both T2 and T3 (**Figure 1**).

Compared to anti-PCSK9 mAb therapy, patients treated with inclisiran showed a decrease in both LDL-C and PCSK9 plasma levels (from 92±28 mg/dL to 44±26 mg/dL and from 691±187 ng/mL to 212±63 ng/mL, respectively) one month after the first inclisiran injection (T1) (n=5; **Figure 2**). The calculated percentage of association between PCSK9 and LDL at T1 was comparable to that at T0. After three and nine months of inclisiran treatment (T2 and T3, respectively), the percentage of PCSK9 bound to LDL remained unchanged, with no further variations in LDL-C and PCSK9 levels.

Discussion

Several studies have shown that PCSK9 is associated with LDL in plasma [5, 17, 18]. To date, the nature and the physiologic role of the PCSK9 associated with LDL and other lipoproteins, such as Lp(a) [18], remains unclear. Some observations suggest that LDL-bound PCSK9 is the more functional form of this protein, as the interaction with an LDL particle protects PCSK9 from cleavage by furin and the protein remains bound to the particle in its active form [19]. On the other hand, in vitro studies showed that the addition of recombinant PCSK9 to LDL reduces the affinity of PCSK9 for LDLR, suggesting that LDL-bound PCSK9 is a less functional form of plasma PCSK9 [5, 20].

PCSK9 inhibitors (monoclonal antibodies and siRNA) have been

Table 1 | Report of total cholesterol (TC), HDL-C, LDL-C and TG in plasma patients before treatments.

Subjects	TC (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	TG (mg/dL)
Patients with mAbs (N=5)	165±15	40±7	97±9	141±48
Patients with siRNA (N=5)	163±35	46±11	92±28	124±44

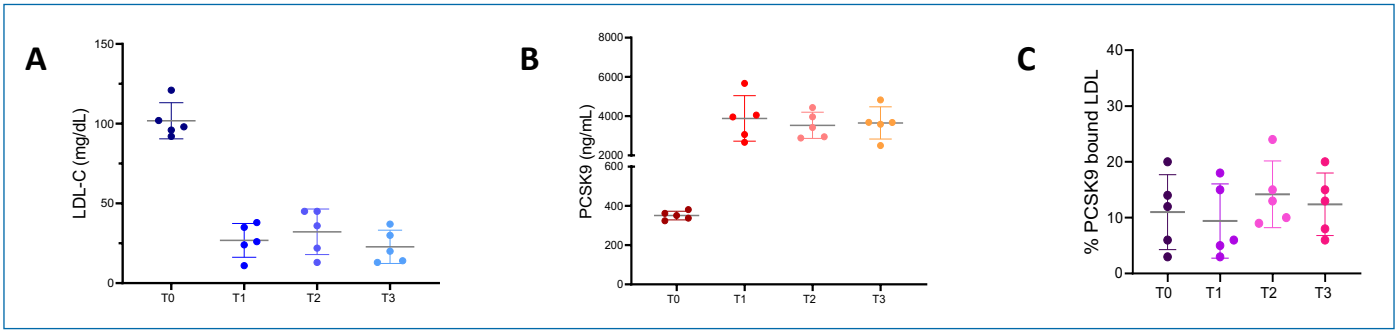

Figure 1 | Comparison among (A) LDL-C and (B) PCSK9 levels before (T0) and after (T1, T2, T3) anti-PCSK9 mAbs administration (n=5 for each group; p<0.005). (C) The percentage of circulating PCSK9 bound to LDL during the therapy (values are means±standard errors).

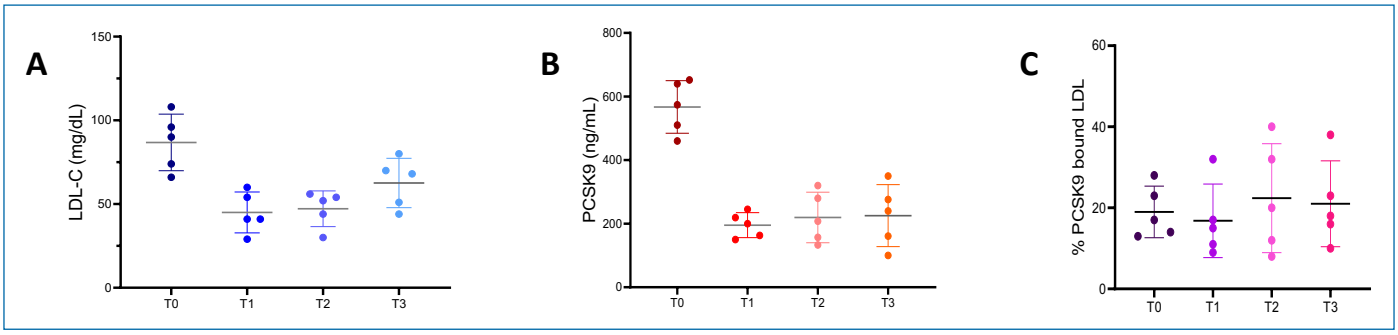

Figure 2 | Comparison among (A) LDL-C and (B) PCSK9 levels before (T0) and after (T1, T2, T3) inclisiran administration (n=5 for each group; p<0.005). (C) The percentage of circulating PCSK9 bound to LDL during the therapy (values are means±standard errors).

developed and approved for the treatment of hypercholesterolemia and are used in patients who need substantial reductions in their LDL-C levels to lower cardiovascular risk. In the present study, we aimed to investigate the association between PCSK9 and LDL particles in patients treated with two different anti-PCSK9 approaches.

According to the results from clinical trials [9, 10], we have demonstrated that both monoclonal antibodies and inclisiran reduce LDL-C levels by 50 to 60%. On the other hand, we clearly show that plasma PCSK9 levels differ significantly between the two therapies. In fact, mAbs increase plasma PCSK9 levels by up to 10-fold, which is due to the large amount of PCSK9 bound to the antibodies, whereas inclisiran reduces plasma PCSK9 protein levels by about 70%.

We found that while LDL-C levels were significantly reduced with both treatments, circulating PCSK9 levels behaved as expected (increased with the mAbs and decreased with inclisiran), while the percentage of PCSK9 bound to LDL did not vary from baseline and remained constant during the treatment period. Nevertheless, a relatively large amount of PCSK9 remains bound to LDL, especially after treatment with monoclonal antibodies. Whether this is of physiological relevance remains to be addressed. The nature of this association is currently being analysed to determine its status and whether the bound PCSK9 is structurally different from the unbound form. Preliminary observations from our ongoing study suggest that it is not the monoclonal antibody-bound form of PCSK9 that binds to lipoproteins, but the free form. Furthermore, we found that the lipoprotein-bound PCSK9 is not in the cleaved form (unpublished data). The mature form of PCSK9 (62 kDa) is believed to be more effective than the furin-cleaved form (55 kDa) in degrading the LDLR [21]; through western blotting analysis, we observed that the mature specifically associates with the LDL subfraction. This observation supports a previous finding that the PCSK9 species associated with LDL is primarily the intact heterodimer form, whereas the free PCSK9 (non-LDL-bound) is primarily in the furin-cleaved conformation [22]. It is tempting to speculate that the PCSK9-LDL-bound form can explain the biological function of LDLR, but further investigations are needed. It is worth noting that all subjects had previously been treated with statin therapy; therefore, the concentration of PCSK9 protein was elevated. In addition, the distribution of PCSK9 and its binding to lipoproteins may be influenced by prior therapy. Further studies on subjects before therapy are underway.

Our previous studies have investigated the interaction between PCSK9 and LDL [16], showing that high salt concentrations disrupt this binding, suggesting a non-covalent interaction. Moreover, our studies have explored the type of lipoproteins bound to PCSK9, uncovering a preference for a specific LDL subfraction. This particular subfraction, resembling remnant lipoproteins, exhibits increased buoyancy compared to mature LDL, characterised by enriched levels of apoE and apoCs, alongside elevated triglyceride content (unpublished data). This observation led us to hypothesise that PCSK9 may enter the bloodstream in association with VLDL, which are then metabolized to IDL, which could explain our finding. This possibility is currently being investigated in a specifically designed study. Overall, the association of lipoproteins with PCSK9 is thought to influence the PCSK9 activity towards the LDL receptor [23], which calls for further investigation into the potential biological significance of this LDL subfraction.

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Authorship and author contribution statement

SM wrote the MS and critically revised the data; VP and FME performed the laboratory work and collected all data; AP critically revised the data and wrote the MS; LG critically revised the data and read the MS; ALC designed the study, critically revised the data and wrote the MS.

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Conflict of interest

AL Catapano reports consulting fees/lecturing fees from Akcea, Amgen, Amryt, Sanofi, Esperion, Kowa, Novartis, Ionis Pharmaceuticals, Mylan, Menarini, Merck, Recordati, Regeneron Daiichi Sankyo, Genzyme, Aegerion, and Sandoz. The remaining authors have nothing to disclose.

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CONFERENCE REPORT

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The IX Spring Meeting of Young Researchers of the Italian Society of Diabetology (SID), the Italian Society of Arterial Hypertension (SIIA), the Italian Society of Internal Medicine (SIMI), the Italian Society of Cardiovascular Prevention (SIPREC) and the Italian Society for the Study of Atherosclerosis (SISA), entitled "*Research drives us crazy",* was held in Rimini on February 25-27, 2024. The Congress was organized by young researchers from the aforementioned scientific societies working in the cardiometabolic field. The Congress hosted five sessions promoted by the five societies, addressing hot topics in the prevention and treatment of cardiometabolic diseases.

More than one hundred young researchers had the opportunity to discuss their scientific work in dedicated oral and poster sessions. In this conference report, we offer an overview of the key topics covered in the presentations at the meeting.

The meeting began with an insightful session organized by SIPREC, dedicated to the role of nutraceuticals in the cardiovascular field. This session addressed evidence from both basic research and clinical studies. Although several novel pharmacological treatments have been developed over the last 20 years to slow the progression of cardiovascular diseases, prevention remains crucial. Nutraceuticals play an important role in pursuing this goal. Nutraceuticals, derived from the terms "nutrition" and pharmaceuticals", are defined as foods or parts of foods that have health-promoting effects ("pharmaceutic properties"). These include polyphenols, carotenoids, polyunsaturated fatty acids, and natural peptides (1). Although guidelines for the treatment of cardiometabolic disorders recognize the role of nutraceuticals in the prevention of atherosclerotic cardiovascular disease (ASCVD), they also stress the weakness of evidence and the lack of concordance between studies. Therefore, caution is required when interpreting the results of randomized controlled trials (RCTs). In recent years, nutraceuticals have been shown to exert important protective cardiovascular effects (2). Albino Carrizzo discussed the molecular mechanisms underlying the biological properties of nutraceuticals, dietary supplements and functional foods leading to improved endothelial function, increased nitric oxide release, reduced production of reactive oxygen species and inflammatory markers, decreased levels of lipoproteins, and inhibition and slowing of the atherosclerotic process (3).

The clinical rationale for the use of nutraceuticals was discussed by Federica Fogacci. She summarized the available evidence on the action of nutraceuticals on plasma lipids, glucose metabolism, liver enzymes, HOMA-IR and body fat and explained their potential implications in clinical practice for the treatment of cardiovascular risk factors and diseases. Among these, red yeast rice is one of the nutraceuticals with the most solid clinical evidence of efficacy, with a dose-dependent lipid-lowering efficacy supported by large meta-analyses of RCTs (4).

The use of well-studied nutraceuticals with defined molecular targets, taken at appropriate dosage and over an adequate period, can contribute to reducing individual cardiovascular risk. However, it is important to emphasize that the use of nutraceuticals should never be considered a substitute for pharmacological treatment.

Day 2 of the congress began with a session on regenerative medicine, exploring innovative approaches for cardiovascular health. Post-ischemic heart failure remains a leading cause of death and disability worldwide, making regenerative therapies a promising approach. In the session organized by SISA, Paola Cattaneo summarized the major limitations in cardiac regenerative therapies: the inability of adult cardiomyocytes to proliferate and the formation of fibrotic scars that replace damaged cardiomyocytes.

The hearts of newborns retain the ability to regenerate, leading researchers to study embryonic mechanisms governing cardiac development and the reprogramming factors that facilitate the transition to adult cardiomyocytes. Potential targets to reverse maturation may include epigenetic enzymes, which are differentially expressed during differentiation (5). DOT1L, an epigenetic enzyme catalyzing methylation of lysine 79 of histone 3, regulates specific gene regulatory networks required for left ventricular morphogenesis and postnatal cardiomyocyte cell cycle withdrawal during embryonic cardiogenesis. Studies in cardiomyocytes and mice suggest that transient inhibition of DOT1L in postnatal hearts could be a strategy to promote the re-acquisition of mitotic potential in cardiomyocytes (5).

Following this, Mario D'Oria provided an overview of the clinical applications of regenerative medicine, with a particular focus on vascular medicine. Chronic limb-threatening ischemia (CLTI) represents the most severe form of peripheral arterial disease affecting the lower limbs; it manifests with rest pain or tissue loss due to an anatomically complex multilevel atherosclerotic burden, often associated with diabetes mellitus. Therefore, it poses a significant threat to the limbs, decreases the quality of life and carries a dramatic cardiovascular risk. Endovascular treatment is currently established as a first-line option for the revascularization of CLTI patients, either alone or in combination with open surgery in hybrid interventions.

Despite the tremendous advancements in the field of revascularization techniques and technologies, which currently allow limb salvage in a significant proportion of patients, some patients fail in all attempts to achieve good in-line arterial flow to the foot and therefore usually face major amputation as the only therapeutic option to control pain or infection. These patients, also known as "no-option CLTI", may benefit from alternative regenerative medicine approaches to (re)establish sufficient distal perfusion to maintain limb viability and reduce pain (6). Despite some clinical trials that have investigated the use of regenerative medicine approaches to achieve (neo)vascularization in no-option CLTI patients, results so far remain highly heterogeneous. There are several barriers to the successful implementation of regenerative medicine methods in this area, such as the heterogeneous cellular sources that have been investigated, different protocols for delivery routes and difficulties with the assessment of cellular homing, the correct identification of the subsets of patients who would benefit most, and uncertainty about the perfusion threshold (and its duration) required to achieve sustained and meaningful clinical benefit. Newer studies are currently being undertaken and their results may reveal whether regenerative medicine will ultimately be a viable therapeutic alternative or whether it is far from the bedside.

The subsequent session, organized by SIIA, focused on the impact of extreme conditions on the cardiovascular system. Giovanni Vinetti and Grzegorz Bilo addressed the topic using the high-altitude (HA) as a model to study integrated cardiovascular regulation in health and disease, whether in natural environments or environmental chambers such as the recently-built terraXcube at Eurac Research, in Bolzano. Reduced alveolar oxygen tension induces generalised pulmonary arteriolar vasoconstriction, which increases pulmonary arterial pressure and right ventricular end-diastolic volume. In the systemic circulation, hypoxemia-induced vasodilation, chemoreflex-induced sympathetic outflow to the heart and vessels, and cold cutaneous vasoconstriction coexist. Acutely, this results in a decrease in total peripheral resistance and an increase in resting and submaximal exercise cardiac output (exclusively via heart rate), with resting arterial pressure unchanged or slightly increased (7). Chronically, the increase in haemoglobin concentration restores resting and submaximal cardiac output to sea-level values, while arterial pressure remains elevated due to increased blood viscosity and decreased local vasodilatory demand. The increased erythropoiesis compensates for the loss of plasma volume only after one month or longer exposure

to hypoxia. Therefore, total blood volume is reduced during most of the stay, as are cardiac filling pressures and left ventricular end-diastolic volume. Maximal exercise capacity is reduced both acutely and chronically, with exhaustion occurring at a lower cardiac output, heart rate, and vagal withdrawal compared to normoxia. In patients with coronary atherosclerosis, HA may trigger myocardial ischemia due to the combined effects of increased afterload, lower oxygen supply and unfavourable coronary haemodynamics (relatively reduced diastolic perfusion time because of a higher heart rate). Nonetheless, the available data suggest that acute HA exposure is safe in patients who underwent coronary revascularization even in the presence of residual noncritical lesions (8). In hypertensive patients, an increase in blood pressure may occur at HA, but this is generally mild if blood pressure is well-controlled at low altitude and treatment adjustment is only occasionally needed (9). Overall, current clinical recommendations suggest that, with some exceptions (e.g., pulmonary hypertension or cyanotic congenital heart disease), HA exposure is not contraindicated in patients with pre-existing cardiovascular disease in stable clinical conditions. Such patients should be carefully evaluated on an individual basis and, in general, should continue to take their existing medications while on HA.

Day 2 ended with a session organized by SIMI, delving into the promises and perils of artificial intelligence (AI) in cardiovascular diseases. Luca Palazzolo introduced the basic concepts of AI. He formally defined the term, starting from the Turing Test, which asks whether it is possible to distinguish between a hypothetical human or artificial interlocutor (10). Subsequently, he reflected on algorithms capable of simulating human dialogues, particularly highlighting the dialogue between Eliza and Parry, and discussed the use of AI in complex games such as chess. Basic notions of machine learning and deep learning were then provided, briefly touching upon the conception and architecture of neural networks and their application in design and content creation. The last part of the presentation involved a dialogue with ChatGPT, aiming to present AI as a statistical model capable of formulating sentences "connected by logical/mathematical operators based on probabilistic calculations", thus defining how it does not experience emotions or feelings but can still mimic these concepts. During the discussion, the audience asked the question "Can AI replace the professional figure of a doctor?". Both speakers firmly denied this possibility, asserting that AI can only support decision-making, which must still rely on personal and professional experience and knowledge.

Giulio Francesco Romiti further explored the potential of AI in enhancing the diagnosis and prognostic evaluation of ASCVD (11). For example, AI can help physicians identify the 30% of patients with coronary occlusion that escape diagnosis. However, it was underlined that AI is not a flawless machine, but only provides a probability estimate and therefore cannot replace clinical judgment. Moreover, critical aspects were highlighted - from the challenges in integrating AI into current clinical workflows to the imperative for comprehensive external validation of existing models to ensure their reliability. The need for rational use of AI in medicine was emphasized, and a specific focus was also dedicated to the diagnosis and screening of atrial fibrillation and the resulting thromboembolic risk stratification. Atrial fibrillation is the most common arrhythmia and often occurs asymptomatically, posing challenges for diagnosis and treatment. AI systems exhibit commendable performance in identifying atrial fibrillation and stratifying thromboembolic risk, thus offering a significant advance in CVD management.

The last scientific session of the meeting, organized by SID, was dedicated to sodium-glucose transport protein 2 (SGLT2) inhibitors, also known as gliflozins, in primary prevention. Valentina Genchi and Renata Risi summarized the preclinical and clinical evidence supporting the use of SGLT2 inhibitors.

SGLT2 inhibitors (SGLT2i) belong to a class of medications used in the treatment of type 2 diabetes. These drugs work by blocking the SGLT2 cotransporter, a protein found primarily in the renal tubules and responsible for reabsorbing filtered glucose in the kidneys and returning it to the bloodstream. These drugs increase the urinary excretion of glucose, thereby reducing blood glucose levels. This mechanism of action is independent of insulin and leads to an effective reduction of blood glucose, particularly in patients with type 2 diabetes, which is characterized by insulin resistance and a reduced ability to utilize glucose. In addition to their hypoglycemic effect, SGLT2i have shown pleiotropic effects that collectively exert beneficial effects on the kidneys, liver, adipose tissue and heart (12). Clinical trials and real-world data have shown that SGLT2i reduce all-cause mortality, composite outcomes of CV mortality, MACE, and hospitalization for heart failure, regardless of diabetes status, glycosuric activity, and previous CV disease (13). SGLT2i also prevent diabetes-related renal tubular damage and myocardial dysfunction by tackling apoptotic, inflammatory, and fibrotic pathways. They also improve renal filtration and reduce the progression of albuminuria in all classes of renal filtrate. Finally, SGLT2i have shown promising effects in resolving hepatic steatosis and reducing fat mass by stimulating the browning of white adipose tissue. Future challenges will be to tailor treatment to those patients who may benefit most from the use of gliflozin.

The congress, following the tradition, featured an unconventional session, which in this edition was dedicated to a debate on error and cognition in medicine. In this edition, Fabrizio Elia led the discussion on the presence of diagnostic errors in medical practice, despite technological advancements, and physicians' reluctance to acknowledge them. He explored the two modes of human reasoning, fast and analytical, highlighting how cognitive errors can impact clinical practice.

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SELECTED ABSTRACTS

Congress Abstract, Spring Meeting 2024, Young Researchers

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Determinants of early subclinical systolic dysfunction in patients with type 2 diabetes

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Aim: Type 2 Diabetes (DM2) is a risk factor for the development of heart failure (HF). Global longitudinal strain (GLS) is more sensitive than ejection fraction (EF) in diagnosing subclinical left ventricular systolic dysfunction (LVSD). Metabolic-associated fatty live disease has been involved in the development of subclinical LVSD-GLS. However, determinants of subclinical LVSD-GLS in DM2 remain poorly known. Our aim was to identify variables associated with altered GLS values in a cohort of DM2 individuals without heart disease and with normal EF.

Methods: The study was performed on DM2 patients (n=150) recruited in the TESEO cohort study with available data on GLS (Speckle Tracking Echocardiography, Epiq CVx Philips), hepatic steatosis (CAP), and liver stiffness (LS) (Fibroscan). Subjects with symptomatic HF, cardiovascular disease (CVD), other heart diseases, EF<50%, eGFR<30 ml/min/1.73 m^2, alcohol abuse, non-metabolic liver disease, and hepatic cirrhosis were excluded. Multiple regression and logistic regression analyses were used to identify GLS determinants and variables associated with subclinical LVSD-GLS (GLS≥-18%).

Results: Recruited subjects (age 61.39±7.89 years, male 57.3%) had a short DM2 duration (3.93±5.06 years) and good metabolic control (HbA1c 6.57%±1.00). Subclinical LVSD-GLS was present in 20% of subjects. Patients with LVSD-GLS had significantly higher LS values (5.77±1.75 vs 4.94±1.25, p=0.003). In multivariate regression analysis, LS values were a significant determinant of GLS, independent of age, waist circumference, diabetes duration, blood pressure, and e' lateral. In logistic regression analysis, LS was associate with a 61% (95% CI 1.15-2,25) increased OR of LVSD-GLS independent of age, gender, WC, diabetes duration, blood pressure, ACR, LVH, and e' lateral.

Conclusions: This study demonstrated that LS is independently associated with LVSD-GLS in DM2 patients with normal HF and without CVD. Abnormal LS values may identify a subgroup of DM2 patients at higher risk of symptomatic HF, who may benefit from closer clinical and echocardiographic monitoring.

Real-world evidence evaluation of LDL-C among hospitalized patients: a population-based observational study in the timeframe 2021-2022

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Aims: European registries and retrospective cohorts highlighted the lack of low-density lipoprotein-cholesterol (LDL-C) goal achievement in many very high-risk patients. Hospitalized patients are often frail, and frailty is associated with all-cause mortality and cardiovascular mortality. Aim of this study is to evaluate LDL-C levels in a Real-World setting of inpatients, identify cardiovascular risk categories and highlight treatment gaps in the implementation of LDL-C control.
Methods: This retrospective, observational study included all the adult patients admitted at an Italian hospital between 2021-2022 and with LDL-C values available during hospitalization. Disease-related real-world data were collected from Hospital Information Systems using automated data extraction strategies and through the implementation of a patient-centered data repository (the Dyslipidemia Data Mart). Assessment of cardiovascular risk profiles, LDL-C target achievement according to the 2019 ESC/EAS guidelines and lipid-lowering therapies (LLT) use were performed.
Results: 13,834 patients were included: 17.15%, 13.72%, 16.82% and 49.76% were low (L), moderate (M), high (H) and very high-risk (VH) patients, respectively. The percentage of in-target patients was progressively lower moving towards worse categories (78.79% in L, 58.38% in M, 33.3% in H and 21.37% in VH). Among LLT treated patients in VH category, in-target are 28.48%; 47.6.% in H, 69.12% in M and 68.47% in L. The impact of monotherapies and combination therapies on target achievement was also analyzed.
Conclusions: This study depicts LDL-C control among an entire population of inpatients, highlighting relevant gaps especially in VH category. Future efforts must aim to reduce the cardiovascular risk of these subjects.

Using AI to identify left ventricular ejection fraction from the ECG: The SOLOMAX (SOciaL NetwOrk of MedicAl Experiences) project

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Aim: The interest in machine learning-based algorithms in the cardiovascular field is rapidly growing, especially for diagnostic and prognostic purposes. Recent evidence has demonstrated that certain electrocardiographic (ECG) parameters are predominantly associated with systolic function, estimated as left ventricular ejection fraction (LVEF) by echocardiography, albeit with still relatively low accuracy.
Consequently, this study aims to develop an AI-based model capable of predicting LVEF from ECG data in an Italian population.
Methods: Within the SOLOMAX project, we collected paired ECG-Echocardiography exams from 105 patients (64.82 ± 16.02y; 62.86% male). Precisely, we excluded patients with atrial fibrillation at the time of the ECG, PMK or electrostimulated rhythm, valve prostheses, previous cardiac surgery, O2 therapy or COPD, previous ablation or invasive electrophysiology procedures, currently hospitalized for Takotsubo or ACS, heart failure exacerbation, inotropic therapy, ACS over the last 3 months. We recorded anthropometric, clinical, biochemical, ECG, and Echocardiography parameters. The collected data was studied using AI-based techniques to create a new model to predict LVEF from ECG. Using an approach based on evolutionary algorithms, genetic programming was used. This approach solves a symbolic regression problem through genetic algorithms and provides a mathematical model of the relationship between ECG parameters and LVEF. The formula obtained was then used to build a simple explainable classifier, which provides a global interpretation of the link between ECG parameters and LVEF.
Results: The performance of the proposed approach and the reliability of the results were assessed using the k-fold cross-validation method and by estimating standard metrics derived from the confusion matrix associated with a binary classifier, that is, accuracy, sensitivity, specificity, precision, and F-Measure. The proposed approach consistently demonstrated its ability to distinguish patients with preserved LVEF from those with reduced LVEF. Each metric averaged across all experiments scored approximately 95%. Furthermore, in the expression generated by the AI model, the axes of the P, QRS, and T waves play a prominent role, as they are likely to provide a better interpretation of the three-dimensional cardiac geometry and, consequently, cardiac function.
Conclusions: AI applied to ECG data can be used to create cost-effective diagnostic and predictive tools for assessing LVEF. Indeed, the obtained formula highlights the relationship between ECG parameters and LVEF, as well as its complexity, which can aid in detecting heart diseases.

Metabolic dysfunction-associated steatotic liver disease in people with HIV is associated with lower BMI and more liver fibrosis compared to the uninfected population

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Aim: People with HIV (PWH) are at high risk of metabolic dysfunction-associated steatotic liver disease (MASLD), defined by the presence of hepatic steatosis plus any among overweight, diabetes, hypertension, or dyslipidemia. There are limited data whether MASLD in PWH differs in clinical presentation from MASLD in the uninfected population. Aim: to compare the severity of metabolic and hepatic dysfunction between MASLD patients with and without HIV.
Methods: 212 consecutive HIV mono-infected patients with MASLD at McGill University in Montreal were compared to a sex and age matched MASLD HIV negative control group at Policlinico Hospital in Milan. Fibroscan with controlled attenuation parameter (CAP) was used to define MASLD (CAP≥248 dB/m), severe MASLD (CAP>280 dB/m), and significant liver fibrosis (liver stiffness measurement>7.0 kPa).
Results: PWH with MASLD presented lower median BMI (28[25-31] vs 29[27-32] Kg/m2, p=0.002) and lower prevalence of obesity (26% vs 44%, p<0.001) compared to MASLD uninfected patients, along with a lower prevalence of hypertension (21% vs 38%, p<0.001). The prevalence of dyslipidemia (41% vs 26%, p<0.001), hypertriglyceridemia (26% vs 9%, p<0.001) and low HDL cholesterol (34% vs 15%, p<0.001) was higher in MASLD patients with vs without HIV. No difference in cardiovascular events and diabetes prevalence was observed between the two groups. Regarding liver disease, PWH with MASLD had lower prevalence of severe MASLD (54% vs 74% p<0.001) but higher prevalence of significant liver fibrosis (15 vs 7%, p=0.03) compared to MASLD uninfected patients. After adjustment, HIV positivity was an independent factor associated with significant liver fibrosis (figure).
Conclusions: Despite having lower BMI, PWH with MASLD have a more severe hepatic presentation and atherogenic lipid profile than MASLD uninfected patients. HIV positivity seems to be independently associated with significant liver fibrosis. Screening and follow-up for MASLD and liver fibrosis is recommended in PWH, even if they are lean.

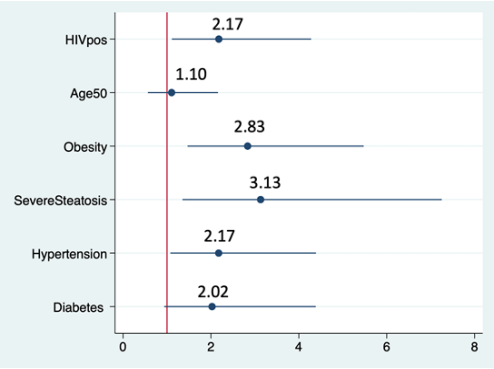

Figure Multivariable regression analysis of factors associated with significant liver fibrosis (adjusted OR with 95% CI).

The aging of neutrophils is actively involved in the metabolic consequences of high fat diet

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Aim: The epigenetic modifications induced by High Fat Diets (HFDs) in long-living hematopoietic cells have been well described, but whether they affect the "aging" of neutrophils, characterized by far shorter half-life, is less clear. Neutrophils "age" through a reciprocal regulation of CXCR4, promoting a "fresh" status when leaving the BM, and CXCR2, accelerating their aging in the circulation. We study whether derailed aging exacerbates the metabolic and inflammatory consequences of HFD.
Methods: We immunophenotyped neutrophils and characterized the metabolic responses in physiology (wild-type mice, WT) and in mice with either constitutively aged neutrophils (MRP8 driven conditional deletion of CXCR4; herein CXCR4fl/flCre+) or with constitutively fresh neutrophils (MRP8 driven conditional deletion of CXCR2; CXCR2fl/flCre+), following 20 weeks of HFD feeding (45% Kcal from fat).
Results: CXCR4fl/flCre+ mice display higher plasma triglycerides levels versus WT, despite comparable glucose levels, when monitored during standard feeding. This metabolic difference was exacerbated by feeding mice a HFD for 20 weeks. Indeed, despite a comparable gluco-metabolic profile between CXCR4fl/flCre+ and WT mice, liver damage was increased in CXCR4fl/flCre+, linked to the higher accumulation of CXCR4fl/flCre+ neutrophils in the liver after 20 weeks and two hours after intragastric gavage with olive oil versus fasting. As this finding was not observed after 20 weeks of standard fat diet, these results suggest that HFD feeding redirects aged neutrophil to the liver, resulting in enriched oxidative metabolism and NETosis- and inflammation-related pathways in the liver of CXCR4fl/flCre+ mice.
Conversely, CXCR2fl/flCre+ mice were protected from obesity and insulin resistance, exhibiting a proresolutive phenotype.
In humans, increased plasma levels of Cxcl1 (ligand of CXCR2) correlated with visceral obesity and metabolic syndrome.
Conclusions: Neutrophil aging might contribute to the cardio-metabolic consequences of HFD. This aging could represent a new therapeutic target beyond the current anti-inflammatory therapies approved for the treatment of cardiovascular diseases.

Transcriptional regulation of Hexokinase-2 by BRD4 drives perivascular adipose tissue meta-inflammation in cardiometabolic disease

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Aim: To investigate BRD4-related transcriptional programmes in mouse and human models of cardiometabolic disease.
Methods: Small arteries (0.1-0.3 mm) dissected from visceral fat biopsies from healthy subjects (n=16) and patients with obesity and hypertension (n=16) were mounted on a pressurized myograph to assess the acute ex-vivo effects of BRD4 inhibition on vascular function. Vasorelaxation to acetylcholine and acetylcholine+L-NAME was evaluated, in the presence or in the absence of perivascular adipose tissue (PVAT), at baseline and after incubation with the BRD4 inhibitor RVX-208 and with selective anti-inflammatory and anti-metabolic drugs. A cardiometabolic mouse (high-fat diet+L-NAME supplementation) was orally administered RVX-208 (150 mg/kg) to test in vivo effect of chronic BRD4 inhibition. ROS and nitric oxide were assessed by confocal microscopy; protein and gene expression by Western blot and qPCR. Transcriptional changes upon BRD4 inhibition were investigated by a custom PCR array, confirmed by ChIP, and characterised by metabolomics, lipidomics and mitochondrial swelling.
Results: Endothelial-dependent vasorelaxation and vascular and perivascular TNF-alpha, IL-1beta, IL-6 were altered in cardiometabolic patients and mice. RVX-208 substantially attenuated ex-vivo vascular dysfunction, with an impact greater than anti-IL-1beta, anti-IL-6 receptor and anti-TNF-alpha. The effect was more pronounced in vessels with intact PVAT, suggesting a restoration of the PVAT anti-contractile phenotype. Gene expression profiling in PVAT unveiled hexokinase-2 (HK2) - a glycolytic enzyme implicated in mitochondrial dysfunction and inflammation - as the top downregulated gene by RVX-208 treatment. Increased binding of BRD4 to HK2 promoter in PVAT samples from cardiometabolic mice was confirmed by ChIP assays. Metabolomics assays further validated the findings by demonstrating a glycolytic shift in PVAT under disease conditions. Finally, ex vivo selective inhibition of HK2 rescued vascular dysfunction.
Conclusion: Targeting the deleterious BRD4-HK2 interplay restores cardiometabolic vascular dysfunction via reversal of the PVAT meta-inflammatory shift, highlighting a novel potential target to fight cardiometabolic pandemics.

Extracellular vesicles characterization in patients with hypertrophic cardiomyopathy

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Background: Hypertrophic cardiomyopathy (HCM) is diagnosed according to the presence of morphological and functional traits of the heart, often in the presence of genetic mutations. A specific biomarker assessing the aetiopathology of this condition is lacking. Extracellular vesicles (EVs), small particles released by all cells into biological fluids, hold promise as diagnostic and prognostic tools for cardiac diseases. We aim at characterising plasma-derived EVs isolated from 18 consecutive HCM patients and 13 healthy volunteers (CTR).
Methods: HCM underwent echocardiographic assessment and genetic testing evaluating 200 genes (NGS). EVs were isolated via ultracentrifugation from platelet-free plasma. Quantitative and qualitative assessments of EVs were performed by nanoparticle tracking analysis and transmission electron microscopy. FACS analysis was used to characterize EV subpopulations. Data are expressed as median and interquartile ranges.
Results: Most patients were male (70.8% HCM and 54% CTR) with a median age of 61 (52.5-71) years (HCM) and 47 (44-52) (CTR). Missense mutations in the *MYH7* gene were the most found in HCM. The median maximum wall thickness in HCM was 16 mm (15-19) vs 8 mm (7-9) in CTR. No differences were found in EV concentrations between HCM and CTR, respectively, $3.6*10^9$ EV/ml/cell count ($2*10^9$-$5*10^9$) and $5*10^9$ EV/ml/cell count ($4*10^9$-$6*10^9$). However, EV concentration was positively associated with the sudden cardiac death risk score in HCM (r= 0.63). Among the EVs positive for CFSE (a specific dye for EVs), those released from platelets, progenitor endothelial cells and neutrophils were increased in HCM patients vs CTR, respectively, by 1.7-, 1.5- and 1.1-fold. A strong negative association (r= -0.74) was found between progenitor endothelial cell-derived EVs and the E/E' ratio of diastolic function, a strong predictor of first cardiac events.
Conclusions: HCM patients present a peculiar phenotypic pattern of EVs that associates which diastolic function and sudden cardiac death.

Definition of the metabolic pattern of ANGPTL3 deficient mice on a chow diet and under dysmetabolic conditions

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Aim: ANGPTL3 controls lipid and lipoprotein metabolism through lipoprotein lipase and endothelial lipase inhibition and the prevention of lipoprotein-derived triglycerides hydrolyzation.
Here we present the metabolic profile of ANGPTL3 deficient mice in physiology and during the development of metabolic disorders.
Methods: Angptl3 KO mice (C57BL6/J background) and their littermate controls (wild-type, WT) were fed a chow and a High Fat Diet (HFD, 60%kcal from lipids) for 16 weeks.
During the diet protocol, changes in lipids and lipoprotein profile, under fast, fed, and fast-refeed setting were assessed. The metabolic phenotype was assessed, with a Glucose Tolerance Test (GTT) and an Insulin Tolerance Test (ITT); the lipids absorption profile was assessed with an Oral Lipid Tolerance Test (OLTT).
Results: ANGPTL3 KO mice fed *ad libitum* a chow diet are hypolipidemic (plasma triglycerides levels: 42,42±8,80 mg/dL in ANGPTL3 KO mice compared to 122,02±55,09 mg/dL in WT mice; plasma cholesterol levels: 44,00±9,11 mg/dL in ANGPTL3 KO mice compared to 76,51±15,87 mg/dL in WT mice).
After 16h fasting, ANGPTL3 KO mice on a chow diet are hypolipidemic and display small lipoproteins less rich in cholesterol and triglycerides, as established in humans, and the same holds true for mice fed a HFD diet.
On HFD, ANGPTL3 KO mice gain less body weight, suggesting an improved metabolic profile compared to WT animals.
The hypolipidemia is conserved during all the timepoints of OLTT, both in mice on chow or HFD, suggesting a different lipid management; in spite, no significant differences in the circulating glycaemia has been proved with a GTT after 16h of fasting; likewise, a similar sensitivity to insulin has been outlined with an ITT after 4h of fasting.
Conclusions: This metabolic profiling of ANGPTL3 KO mice on chow diet or HFD highlights that these mice are hypolipidemic and may have beneficial metabolic features compared to controls.

Cholesterol esterification is hampered in alzheimer's disease and cholesteryl esters composition is consequently altered

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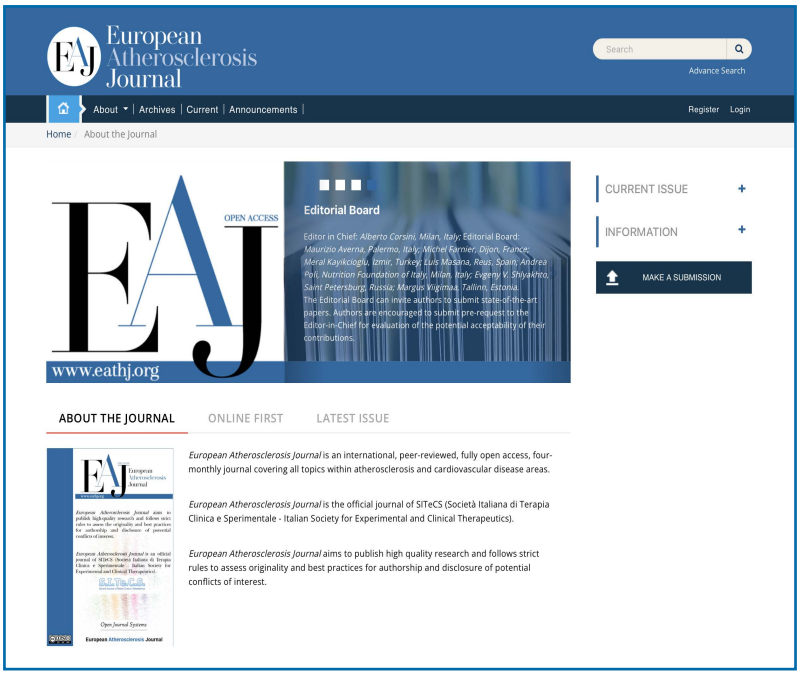
Introduction and Aim: Several epidemiological studies indicate a strong inverse association between the risk of developing Alzheimer's disease (AD) and plasma HDL-C levels. The mechanism by which plasma HDL influence the pathogenesis and progression of AD is still unsolved and since cholesterol esterification is a crucial step in HDL metabolism it could be involved. The purpose of this study was to evaluate cholesterol esterification and HDL subclasses in plasma and cerebrospinal fluid (CSF) of Alzheimer's Disease (AD) patients.
Methods: The study enrolled 70 AD patients and 74 cognitively-normal controls comparable for age and sex. Lipids and lipoprotein profile, cholesterol esterification, and cholesterol efflux capacity (CEC) were evaluated in plasma and CSF using assays set for measurement in plasma, which were appropriately modified for CSF.
Results: AD patients have normal plasma lipids, but significantly reduced unesterified cholesterol and unesterified/total cholesterol ratio. Lecithin:cholesterol acyltransferase (LCAT) activity and cholesterol esterification rate (CER), two measures of the efficiency of the esterification process, were reduced by 29% and 16%, respectively, in plasma of AD patients. Plasma HDL subclass distribution in AD patients was comparable to that of controls, but the content of small discoidal preβ-HDL particles was significantly reduced. In agreement with the reduced preβ-HDL particles, cholesterol efflux capacity mediated by the transporters ABCA1 and ABCG1 was reduced in AD patients' plasma. The CSF unesterified to total cholesterol ratio was increased in AD patients, and CSF CER and CEC from astrocytes were significantly reduced in AD patients. In the AD group, a significant positive correlation was observed between plasma unesterified cholesterol and unesterified/total cholesterol ratio with Aβ1-42 CSF content.
Conclusions: Taken together data indicate that cholesterol esterification is hampered in plasma and CSF of AD patients, and that plasma cholesterol esterification biomarkers (unesterified cholesterol and unesterified/total cholesterol ratio) are significantly associated to disease biomarkers (i.e., CSF Aβ1-42).



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