Rationale of the FIBROTARGETS study designed to identify novel biomarkers of myocardial fibrosis

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Abstract

Aims Myocardial fibrosis alters the cardiac architecture favouring the development of cardiac dysfunction, including arrhythmias and heart failure. Reducing myocardial fibrosis may improve outcomes through the targeted diagnosis and treatment of emerging fibrotic pathways. The European-Commission-funded ‘FIBROTARGETS’ is a multinational academic and industrial consortium with the main aims of (i) characterizing novel key mechanistic pathways involved in the metabolism of fibrillary collagen that may serve as biotargets, (ii) evaluating the potential anti-fibrotic properties of novel or repurposed molecules interfering with the newly identified biotargets, and (iii) characterizing bioprofiles based on distinct mechanistic phenotypes involving the aforementioned biotargets. These pathways will be explored by performing a systematic and collaborative search for mechanisms and targets of myocardial fibrosis. These mechanisms will then be translated into individualized diagnostic tools and specific therapeutic pharmacological options for heart failure.

Methods and results The FIBROTARGETS consortium has merged data from 12 patient cohorts in a common database available to individual consortium partners. The database consists of >12 000 patients with a large spectrum of cardiovascular clinical phenotypes. It integrates community-based population cohorts, cardiovascular risk cohorts, and heart failure cohorts.

Conclusions The FIBROTARGETS biomarker programme is aimed at exploring fibrotic pathways allowing the bioprofiling of patients into specific ‘fibrotic’ phenotypes and identifying new therapeutic targets that will potentially enable the development of novel and tailored anti-fibrotic therapies for heart failure.

Keywords Myocardial fibrosis; Fibrotic bioprofiles; Heart failure

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prevalent cell type in the heart and are essential not only for normal cardiac function, being involved in the synthesis and deposition of the extracellular matrix (ECM), cell–cell communication with myocytes, and cell–cell signalling with other fibroblasts and with endothelial cells but also for post-aggression repair processes (e.g. myocardial scar formation after infarction). Myocardial fibroblasts respond to several mediators, including pro-inflammatory cytokines (e.g. tumour necrosis factor, interleukins 1 and 6, and transforming growth factor beta), vasoactive peptides (e.g. angiotensin II, endothelin 1, and natriuretic peptides), hormones (e.g. cortisol, renin, aldosterone, norepinephrine, and mechanical stretch), and changes in oxygen availability (e.g. ischaemia–reperfusion). Thus, any dysregulation at the level of these mediators may lead to dysfunctional excessive fibrosis affecting the electrophysiological pathways, mechanical properties, and signalling functions of the heart. Insults to the heart such as pressure or volume overload, ischaemic injury, valvular diseases, and genetic predispositions favour the development and in overloading, ischaemic injury, valvular diseases, and genetic predispositions favour the development and influence the amount of myocardial fibrosis. The latter develops into two distinct patterns: as a scar to replace the loss of cardiomyocytes (referred to as focal reparative fibrosis) and as pervasive fibrosis diffusively invading the myocardial interstitium and the perivascular space (referred to as diffuse fibrosis). While both types of myocardial fibrosis result from a dysregulated collagen turnover (i.e. synthesis exceeds degradation, facilitating the deposition of highly cross-linked, stiff, and degradation-resistant collagen fibres) impairing cardiac function, the excessive accumulation of collagen in the ECM may result from various altered signalling pathways. While fibrosis is essential for cardiac repair, its excessive and aberrant accumulation is deleterious; hence, there is a thin line separating ‘good’ from ‘bad’ fibrosis. The identification and validation of circulating biomarkers showing good correlation with the ‘intracardiac’ mechanistic pathways involved in interstitial fibrosis are essential for developing bioprofiles that could help in identifying patients with ‘bad’ fibrosis and who are likely to benefit from targeted therapeutic agents. However, it is also plausible that different pathological phenotypes result from the interweaving of several altered molecular pathways. Hence, an integrative and comprehensive approach is essential to better understand these fibrotic disease processes. Moreover, specific therapeutic options targeting myocardial fibrosis have failed to demonstrate undisputable efficacy, hence the need for novel therapeutic options.

By design, the FIBROTARGETS project integrates experimental and clinical research with the aim of (i) characterizing novel key mechanistic pathways involved in the metabolism of fibrillar collagen that may serve as biotargets, (ii) evaluating the potential anti-fibrotic properties of novel or repurposed molecules interfering with the newly identified biotargets, and (iii) characterizing bioprofiles based on distinct mechanistic phenotypes involving the aforementioned biotargets targeting a tailored treatment approach. The purpose of this article is to describe the design of the clinical studies addressing the latter objective.

### Study population

Taking advantage of its multinational structure, the FIBROTARGETS consortium has merged data from 12 patient cohorts in a common database available to individual consortium partners. The database consists of >12 000 patients with a large spectrum of cardiovascular clinical phenotypes. It integrates community-based population cohorts (Stanislas and Health ABC), cardiovascular risk cohorts (REVE-1 and REVE-2, ADELAHYDE, R2C2, HVC, REMI, and STOP-HF), and heart failure (HF) cohorts (Leitzaran, MEDIA-DHF, and TIME-CHF). Cohort details are summarized in Table 1 and the Supporting Information, Tables S1, S2, and S3.

Patients from the merged database were clustered into four main phenotypic groups: (i) ‘healthy subjects’, including (but not exclusively consisting of) participants without cardiovascular risk factors or renal dysfunction, (ii) participants at ‘risk’ of developing HF as evidenced by the presence of diabetes and/or hypertension or other cardiovascular risk factors and no overt cardiac dysfunction, (iii) patients with prevalent HF with reduced left ventricular ejection fraction regardless of HF symptoms, and (iv) patients with prevalent HF with preserved left ventricular ejection fraction regardless of HF symptoms.

### Selection of biomarkers of interest

The biomarkers to be studied in the FIBROTARGETS consortium are related to the novel targets studied within the project and reflect various aspects related to myocardial fibrosis and are summarized in Table 2.

A number of molecules involved in the extracellular formation and degradation of collagen type I that is secreted from the heart to the blood stream, and thus detectable in either the serum or plasma by using immunoassay methods, have recently been proposed as biomarkers of diffuse interstitial fibrosis. For instance, serum C-terminal propeptide of procollagen type I (PICP) has a good correlation with histologically proven severe myocardial interstitial deposition of collagen type I fibres and collagen volume fraction ($r = 0.7$) in hypertensive patients with stage C HF. The molecular basis of this biomarker is that the cleavage of PICP from the procollagen type I precursor will determine the ability of the resulting collagen type I molecule to form fibrils. Excessive myocardial collagen cross-linking is associated with HF...
hospitalization in hypertensive patients with HF, and the serum C-terminal telopeptide of collagen type I (CITP) : matrix metalloproteinase 1 (MMP-1) ratio allows identification of patients with increased collagen cross-linking and high risk for HF hospitalization.19 The molecular basis of this biomarker relies on the consideration that a diminished circulating level of CITP (corrected by circulating MMP-1) reflects reduced collagen type I fibre degradation by MMP-1 given that high lysyl-oxidase-mediated cross-linking increases the resistance of the fibre to MMP-1 proteolysis.20 On the other hand, recent clinical data suggest that cardiotoxin 1 (CT-1), a member of the interleukin 6 superfamily, behaves as a profibrotic cytokine. In fact, CT-1 stimulates the differentiation of human cardiac fibroblasts to myofibroblasts and the expression of procollagen type I and III messenger RNAs in these patients.21 In addition, CT-1 overexpression has been found to be associated with increased expression of collagen types I and III in the myocardium of HF patients.21 Moreover, an excess of plasma CT-1 has been associated with both increased serum PICP and increased serum N-terminal propeptide of procollagen type III, a molecule formed during the conversion of procollagen type III into collagen type III by the enzyme procollagen type III aminoterminal proteinase, which is correlated with the myocardial deposition of collagen type III fibres,22 in these same HF patients.21 Conversely, apelin, a small anti-collagen production peptide, has been found to be decreased in the plasma of patients with advanced HF. In addition, in rodent cardiac fibroblasts, apelin has been shown to reduce the production of collagen and the activation of fibroblasts induced by transforming growth factor beta.23 Interestingly, exogenous administration of apelin was able to reduce myocardial fibrosis and improve cardiac function in a rodent model of HF.24

Multiple studies have highlighted the association of inflammation with myocardial fibrosis, and several inflammation-related biomarkers have been identified as predictors of clinical outcome in HF patients.25 For instance, serum galectin 3 has been reported to be a good predictor of dismal events and mortality in HF patients.26 Similarly, soluble ST2 has been reported to be independently associated with cardiovascular mortality in HF patients,27 and growth differentiation factor 15 with mortality and

| Table 1 Clinical characteristics of the merged FIBROTARGETS cohort |
|---------------------------|---------------------------|
| Parameter                     | Available data Merged FIBROTARGETS database (12 922) |
| Demographics                | n (Baseline)              |
| Women, n (%)                 | 12 922 6365 (49)          |
| Age, years                  | 12 921 54 ± 23            |
| BMI, kg/m²                   | 12 419 25.7 ± 5.6         |
| Current smoker, n (%)        | 11 318 2078 (18)          |
| Current alcohol consumer, n (%) | 8859 4461 (50) |
| Medical history              |                          |
| Hypertension, n (%)          | 7443 4906 (66)           |
| Diabetes mellitus, n (%)     | 8445 1624 (19)           |
| Hypercholesterolaemia, n (%) | 4366 1565 (36)           |
| Myocardial infarction, n (%) | 4742 1231 (26)           |
| Heart failure, n (%)         | 6158 1763 (29)           |
| Atrial fibrillation, n (%)   | 3695 655 (18)            |
| Haemodynamics                |                          |
| Systolic blood pressure, mmHg| 12 486 129 ± 21          |
| Diastolic blood pressure, mmHg| 12 484 72 ± 14           |
| Heart rate, b.p.m.           | 12 734 70 ± 13           |
| Blood biology                |                          |
| Total cholesterol (mmol/L)   | 11 681 5.2 ± 1.3         |
| Glucose, mmol/L              | 11 528 5.70 ± 1.91       |
| Haemoglobin, g/dL            | 7286 14.0 ± 1.4          |
| Serum creatinine, μmol/L     | 10 566 86.5 ± 35.6       |
| Blood biomarkers             |                          |
| BNP, pg/mL                   | 1441 36 (13–116)         |
| NT-proBNP, pg/mL             | 1542 2502 (1074–6115)    |
| Echocardiographic parameters |                          |
| LVmass, g                    | 2823 167 ± 77            |
| LVEF, %                      | 4291 57 ± 14             |
| LVEF < 40%                   | 4291 586 (14%)           |
| 40% ≤ LVEF ≤ 50%             | 4291 551 (13%)           |
| LVEF > 50%                   | 3154 3154 (73%)          |
| E/A ratio                    | 3112 1.12 ± 1.60         |

BMI, body mass index; BNP, B-type natriuretic peptide; LVEF, left ventricular ejection fraction; LVmass, left ventricular mass; NA, not applicable; NT-proBNP, N-terminal pro-B-type natriuretic peptide. Continuous variables are described as mean ± standard deviation or, for non-normal variables, as median (first quartile to third quartile). Dichotomous variables are described as number of events (% of available data).
non-fatal events in HF with preserved or reduced ejection fraction.\textsuperscript{28}

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin 2 or oncogene 24p3, is considered as a biomarker of kidney injury because it is rapidly released in response to kidney tubular damage.\textsuperscript{29} However, increased systemic levels have also been reported in patients with HF and myocardial infarction, with these levels being associated with adverse cardiac events.\textsuperscript{30} For example, NGAL is up-regulated in the cardiovascular system in various pathological models such as atherosclerosis,\textsuperscript{31} aortic abdominal aneurysm,\textsuperscript{32} and after myocardial infarction.\textsuperscript{33}

### Non-coding RNAs

Many studies have shown that non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are released into the circulation where they exhibit specific profiles that differ between healthy subjects and diseased patients.\textsuperscript{34} These molecules are increasingly recognized as non-invasive and readily accessible biomarkers for risk stratification, diagnosis, and prognosis of cardiac injury and multiple forms of cardiovascular disease. Several miRNAs have recently been linked to the diagnosis of HF.\textsuperscript{34,35} Based on our screening results as well as literature findings\textsuperscript{36,37} several ‘cardiac remodelling’-associated miRNAs and lncRNAs have been chosen to be tested in the FIBROTARGETS patient populations to determine their use for improving diagnosis of HF, risk and patient stratification, and prediction of individual patient prognosis. Standardized miRNA- or lncRNA-specific PCR-based protocols and normalization procedures are currently being used to achieve this goal.

### Data management

The FIBROTARGETS database was elaborated and is maintained at the Inserm Clinical Investigation Center (CIC1433), Nancy, France. In order to be included in FIBROTARGETS, participating institutions and investigators must comply with the latest local, national, and international rules on data management.
protection, biomedical research regulations, good clinical practices, and the Helsinki Declaration. All participants from each cohort must have provided written informed consent. Informative data relevant to the FIBROTARGETS consortium have been extracted from each cohort dataset. Thus, the merged dataset retains a maximum of information retrieved from each contributing cohort in order to include baseline characteristics of each participant (anthropometrics, medical history and medication use, routine haematological and biochemical measurements, electrocardiographic and echocardiographic variables) as well as follow-up information, whenever available. A data dictionary has been elaborated based on agreed common definitions and criteria enabling classification of clinical events and phenotypes with a particular focus on HF events, left ventricular dysfunction, hypertension, diabetes, atrial fibrillation, myocardial infarction, and chronic kidney disease (see Table 1 and Supporting Information, Tables S1, S2, and S3).

The investigators of each study have also collected follow-up data. By ensuring a large diversity of phenotypes within the FIBROTARGETS population (in terms of age and presence or absence of co-morbidities), the merged database provides a unique tool to evaluate the determinants and utility of profiling patients according to specific fibrotic pathways using candidate biomarkers.

### Statistical and bioinformatics considerations

Predictive models (of the aforementioned phenotypic clusters) using biomarker variables will be developed in three ways: (i) using classical statistical methods (as described above) including selection procedures in the second phase, (ii) using machine-learning tools such as regression trees and random forests, and (iii) using system biological or computational models based on available knowledge of myocardial fibrosis. In addition to the prediction of phenotypic clusters, associations between biomarkers and clinical outcomes will be assessed, including all-cause and cardiovascular mortality and HF hospitalizations. Cox models will be used to assess the associations between biomarkers and outcome.

Further bioinformatics analysis will integrate the obtained FIBROTARGETS biomarker data in the context of previous knowledge. The biomarkers will first be analysed for their interaction with diseases and phenotypes (such as cardiovascular diseases, immune disorders, and cancer, among others), drug interactions, protein–protein interactions, and their predicted targeting by miRNAs. Next, correlation network modules will further refine the measured biomarkers in clusters that correlate with specific clinical phenotypes and pre-measured metabolites (such as lipid metabolism). Finally, this cluster analysis will evolve in cluster enrichment for specific biological processes—such as leukocyte migration and fibroblast proliferation—using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathways. In addition to classical biostatistics, this interaction, network, and clustering analysis will place the biomarkers in their biological context for improving selection of biomarkers, understanding their disease context, and refining patient stratification.

A matched case–control design will be chosen to assess the associations between candidate biomarkers and the four phenotypic clusters (as defined above).

Cases from the three patient clusters will be matched with a control from the ‘healthy subjects’ cluster. The distribution of biomarker levels in ‘healthy’ subjects and in patients will be described, and reference values established for each biomarker, with influence of age and gender. The association between candidate biomarkers, disease clinical phenotypes, and outcome will be assessed with appropriate statistical methods, using continuous or categorical variables, including conditional logistic regression adjusted for a set of confounders. The influence of common co-morbid conditions (e.g. diabetes, hypertension, and obesity) will be taken into account in multivariate analyses. In each instance, sensitivity and specificity measurements of the predictions will be quantified.

Power calculations will be performed solely based on the comparison of biomarkers across the four clusters. In the first phase of the analysis, to account for the multiple comparisons and biomarkers, a two-sided alpha of 0.01 will be assumed for sample size calculation. In order to achieve a statistical power of 90% to detect a minimum difference of an odds ratio of 1.35 per 1 SD of the biomarker, the minimum sample size would be 750 consisting of 250 cases and 500 controls.

In the second phase of the analysis, the most promising candidate biomarkers will be tested using a two-sided alpha of 0.001, including 600 patients (200 cases and 400 controls) to provide a 90% power to identify a difference of 1.50 odds ratio per 1 SD of the biomarker. In total, for the first and second phases of the analysis, 450 cases and 900 controls would be required for a total sample size of 1350 patients.

### Baseline characteristics of the study population

The characteristics of the main cohorts are described in Table 1 (‘merged’ dataset) and Supporting Information, Tables S1 (population/‘healthy’ cohorts), S2 (cardiovascular risk cohorts), and S3 (HF cohorts).
The FIBROTARGETS population consists of 12,922 individuals, 49% of whom are of female sex. The mean ± standard deviation (SD) age is 54 ± 23 years with a mean ± SD body mass index of 25.7 ± 5.6 kg/m². Sixty-six per cent of the individuals present a diagnosis of hypertension, 19% had diabetes mellitus, and 29% had HF.

Additionally, individuals were classified based on the four selected risk factors (hypertension, obesity, diabetes, and history of HF), allowing the establishment of subgroups based on disease/risk factors, as represented in Figure 1.

### Outcomes

The available outcomes for each study population are described in Table 3. Outcomes in each study cohort were adjudicated by independent committees.

### Discussion

Therapeutic developments conducted in the last decades have led to a substantial decrease in mortality in HF with reduced ejection fraction. Therefore, demonstrating a therapeutic benefit on top of existing therapies in the logic of ‘one-size-fits-all’ trials has become increasingly difficult and cost demanding. As a consequence, in the last three decades, landmark HF trial sizes have progressed from a few hundred to many thousand patients.40,41 Operational obstacles and unbearable costs are major challenges for the successful implementation and completion of such large trials.42,43 Even with pre-specified subgroup analysis, regulators do not accept testing for responder profiles due to the risk of bias. Substantial warnings and approval deferrals have already been performed on the basis of subgroup results.44,45 Precision medicine has emerged as one possible solution, aiming at identifying the different strata within a disease based on a deeper understanding of the mechanisms underpinning these strata. Accordingly, biomarker-based stratification will likely enable the development of disease-specific diagnostic tools and treatments. The FIBROTARGETS programme is based on the hypothesis that intervention on novel fibrosis-related targets involved in the processes of fibroblast differentiation into myofibroblasts, on collagen synthesis over degradation balance, and/or on collagen maturation may allow for interstitial repair, thus providing a new strategy for the prevention and treatment of MIF, a key mechanism of cardiac remodelling involved in both the transition to and progression of HF. Multi-panel circulating markers, descriptive of mechanisms involving the proposed novel targets, will be used to screen large-scale population of patients and to stratify the latter based on specific ‘fibrotic’ profiles.

In syndromes that are clinically and pathophysiological heterogeneous and frequently associated with several co-morbid conditions such as HF (and HF with preserved ejection fraction in particular), more appropriate biomarkers are needed to improve screening, diagnosis, monitoring, and treatment response. Mitigating or preventing the development of MIF is one of the therapeutic strategies warranting further evaluation. Mineralocorticoid receptor antagonists (MRAs) are the only treatments to date demonstrated to be capable of reducing cardiac fibrosis during HF-induced remodelling.46,47 In fact, post hoc evidence suggests that MRAs are likely to work best in subgroup of patients with elevated cardiac collagen markers46 or with visceral obesity, conditions known to display marked fibrosis. The discovery of more powerful and specific modulators of fibrosis could potentially provide the basis for new drug development and patient selection for future trials.

Previously assessed biomarkers can be classified as collagen quantity (e.g. PICP and N-terminal propeptide of procollagen type III)16 and quality markers (e.g. CITP : MMP-1).16,18 While these biomarker assessments in patients at risk of and with HF have been associated with poor prognosis, only two collagen-derived serum peptides have been shown to be specifically associated with the extent of myocardial fibrosis: PICP and C-terminal propeptide of procollagen type III.16 Beyond these end-products of collagen turnover, FIBROTARGETS aims to explore upstream mechanistic pathways as potential discrete biotargets for novel therapies preventing and/or limiting the progression of MIF.

Figure 1 Venn diagram of the FIBROTARGETS merged cohort, with clustering of individuals with at least one of the listed risk factors. Legend: the merged FIBROTARGETS cohort has been stratified according to co-morbidities [obesity, diabetes, hypertension (HTN), and heart failure (HF)]. Number of individuals displaying from 1 to 4 of these conditions is given within the intersection of corresponding ellipses.
One of the many challenges is that fibrosis is a mechanism involved in a number of overlapping and/or coexisting risk and disease states and co-morbidities such as hypertension, chronic kidney disease, diabetes mellitus, obesity, atrial fibrillation, ischaemic heart disease, and prevalent HF, such that a number of circulating biomarkers can be present in these various conditions. Our hypothesis is that novel ‘mechanistic’ markers of cardiac fibrosis are differentially expressed in various risk and disease states and co-morbidities.

Conclusions

While many biomarkers have been tested, reproducibility and external validity are not sufficiently robust however to derive firm conclusions as to which biomarker(s) can best assess cardiac fibrosis or assess treatment response. Moreover, these biomarkers have not proved to determine senescent cell accumulation in fibrotic myocardial tissue, especially given that myofibroblasts have been identified as the predominant cardiac cell population undergoing senescence and are critical regulators of cardiac fibrogenesis. The FIBROTARGETS programme will explore novel and mechanistic fibrotic targets that will enable the development of disease-specific therapies. The programme includes the investigation of multi-panel circulating markers descriptive of mechanisms involving the proposed novel targets. These will form the basis of the stratification of patients at risk into specific ‘fibrotic’ bioprofiles.

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Conflict of interest

All authors are actively involved in the FIBROTARGETS consortium.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Table S1. Details on the population cohorts.
Table S2. Details on patient cohorts at risk for CV.
Table S3. Details on HF patient cohorts.

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