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PROJECT FINAL REPORT

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Project website Error! Bookmark not defined. **address:** eu-mascara.eu

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1.1. Final publishable summary report

1. Executive Summary

Cardiovascular disease (CVD) is the main cause of death in Europe and worldwide. CVD also leads to disability, hospital admissions and reduced ability to work and therefore affects individual patients, health care systems and societies as a whole. CVD develops slowly and from common risk factors such as high blood pressure, smoking and cholesterol levels. Not all people with these risk factors will develop severe CVD and not all people with severe CVD will have a characteristic risk factor profile. Moreover, not only the severity but also the rate of progression of CVD differ from patient to patient.

The risk of development of CVD and its progression can be estimated from traditional risk factors. However, such predictions are not accurate and may over or underestimate an individual person's risk. The EU-MASCARA project was based on the concept that more individualised risk assessment will be required in order to offer patients the best therapies to prevent and reverse CVD. The project was therefore at the forefront of a development that is now called "precision medicine" – to provide the right treatment to the right patient at the right cost.

The EU-MASCARA Consortium aimed to study if "biomarkers" can help with CVD risk assessment. Biomarkers are features that can be measured, that relate to the disease process and that respond to therapeutic interventions. It should be noted that the term "biomarkers" not only refers to circulating markers that can be measured in blood or urine but that it also includes imaging techniques and functional assessments that can all provide information about a patients' cardiovascular health. The Consortium has therefore looked into a wide range of biomarkers to assess their use for diagnosis of CVD and for prediction of future CVD in the general population and in people who already have risk factors for CVD.

The EU-MASCARA project was built upon four objectives that have been addressed throughout the course of the research programme. First, we studied the relationship of emerging biomarkers with existing CVD. In cross-sectional studies on existing clinical cohorts we studied people with CVD and people at early stages of CVD who were then compared to healthy control subjects. Genetic markers, proteomic markers, metabolomic markers and specific biomarkers of inflammation and cardiac remodelling were studied. Whilst all these markers have a specific role to explain specific steps in the development of CVD it is challenging to find uniform markers that apply to all forms of CVD. In a second step the Consortium has therefore taken most of the biomarkers from the first step into studies where long-term follow-up was available in order to study if the biomarkers can predict the development of CVD. We focussed on heart failure as a model disease as this is a common endpoint of many cardiovascular conditions and associated with significant disability and risk of death. The EU-MASCARA Consortium has developed and validated specific genetic factors (microRNAs) and proteomic factors (namely based on urinary polypeptides) that can be predictive of heart failure; the Consortium has made further developments of other biomarkers and validated them in the context of heart failure but has also studied other cardiovascular conditions including high blood pressure, coronary artery disease and renal diseases.

The third objective was to look comprehensively at all the data and find a way to bring them together in biomathematical models. This "data integration" was based on all biomarker data and also took health economic aspects into account as novel biomarkers are often very expensive to measure. We have also defined a fourth objective of developing new tests for CVD and have made first steps in this direction especially in the area of proteomics. For other biomarkers we have defined the most promising candidates and techniques that can inform future assay development.

2. Summary description of project context and objectives

Cardiovascular disease (CVD) is the major cause of morbidity and mortality in Europe and other Western societies. In Europe, a north-east to south-west gradient in mortality from CVD remains, with age-standardised mortality rates ranging from 731 to 1763 per 100,000. Recently, incidence and prevalence of CVD have also increased in developing countries, making CVD a truly global epidemic. Consequently, CVD is associated with significant costs to health care systems that are expected to rise further in face of the increasing prevalence of cardiovascular risk factors, namely obesity and the metabolic syndrome, in populations worldwide.

A number of cardiovascular risk scores based upon a range of traditional risk factors have been developed and validated. It has, however, been recognised that modifications to risk scores are required to better reflect risk profiles of specific populations. The SCORE model with its global European and national versions is a prime example of such adaptations to different cardiovascular risks across European regions. Despite increasingly precise predictive power on a population basis, risk scores do not necessarily perform well in individual subjects or in groups of subjects who share certain characteristics and may have risk factors but also protective factors that are not represented in risk scores. Some of these are poorly understood or unknown, and may include genetic factors, diet, physical activity and other lifestyle factors. Against this background there is an unmet clinical need for stratified cardiovascular medicine. This will include accurate risk prediction in individual subjects with subsequent targeted preventative therapy. This approach is considered highly cost effective not only due to prevention or delay of onset of CVD related organ damage but also due to the potential of saving on diagnostic procedures drug therapy in subjects at low cardiovascular risk.

From the complexity and heterogeneity of cardiovascular diseases it is clear that single biomarkers will not improve diagnostic and predictive accuracy compared to existing strategies. EU-MASCARA therefore aimed to comprehensively evaluate and validate clinically useful biomarkers and sets of biomarkers in prevention and management of cardiovascular diseases across a spectrum of pathophysiological conditions.

The EU-MASCARA Consortium therefore aimed to validate emerging biomarkers of cardiovascular risk for future clinical use. The project had four specific objectives that were addressed throughout the course of the programme.

Objective 1. To validate the association of emerging biomarkers with cardiovascular phenotypes in cross-sectional disease and population cohorts

The first objective of the project is based on the generally accepted assumption that biomarkers that differentiate overt disease from a healthy state are also suitable to predict development of disease at an early and asymptomatic stage.

A number of different scenarios have been explored throughout the project:

- For quantitative biomarkers such as levels of specific proteins in blood, one would expect that the concentration range in overt disease is far higher than in early and asymptomatic stages of disease but that with sufficiently sensitive assays differences can be measured. Typical examples for this concept are inflammatory markers such as C-reactive protein or cardiac damage markers such as cardiac troponins.
- There are disease markers that are quantitatively different between people at high and low risk. These are often markers that will act in concert with other factors and increase the likelihood for CVD over time. Typical examples include genetic factors that are present from birth (and even before) and do not change throughout the lifespan. Many of the known

genetic factors increase risk only to a minor extent but together with other genetic factors and lifestyle factors such as smoking and diet, they can be powerful determinants of future CVD.

- Disease processes such as vascular stiffening and cardiac hypertrophy are well established intermediate steps in the development of overt CVD. Some of these processes are difficult or costly to be assessed directly as they may for example need advanced imaging techniques that are not readily available to all citizens. There are biomarkers which can mirror these disease processes and could provide information about the heart and the blood vessels without the need for a person to undergo complicated and sometimes invasive tests. Examples come from markers of collagen turnover and from urinary proteomic markers that both provide information in fibrotic processes in the body, in particular in the heart and blood vessels.

The EU-MASCARA Consortium has therefore studied a range of biomarkers in cross-sectional studies with the aims to analyses their relationship with overt CVD but also to study markers of specific disease processes and specific cardiovascular phenotypes. Among the latter are vascular stiffness, (cardiac) left ventricular mass, renal function and functional properties of blood vessels.

In the call text for this programme the European Commission asked for studies of biomarkers that have an already established promising track record and did not ask for the development of new biomarkers. The EU-MASCARA Consortium therefore proposed a two-stage process of biomarker validation: emerging and promising biomarkers were selected based on the partners' expertise and from literature searches and first validated in cross-sectional studies (association with disease and/or subclinical cardiovascular phenotypes); in a second stage a further validation step was proposed in longitudinal studies to assess the predictive value of markers that passed the first validation step (see 3.1.2).

Throughout the course of the project the Consortium has, however, developed a more flexible approach to the originally proposed strict separation between cross-sectional and longitudinal studies. Details on this revised concept are provided in section 3.1.2. It should be noted, however, that this more flexible approach did in no way compromise delivery of the original objectives. Instead, it further enhanced the quality of the outputs and generated additional data and laid a foundation for future collaborative research.

Objective 2. To validate emerging biomarkers as predictors of changes in cardiovascular phenotypes and cardiovascular events in prospective disease and population cohorts

The ultimate aim of EU-MASCARA was the validation of biomarkers that help to predict the development of overt CVD. The Consortium benefited from the availability of cohorts with long-term follow up where baseline blood and urine samples as well as from functional and imaging data which were available.

In the context of disease prediction, it was important for the Consortium to have access to general population cohorts as well as disease cohorts; the clinical needs and the requirements for biomarkers are different in the two scenarios.

- In a population cohort only a relatively small number of individuals will develop CVD during the follow-up period and an even smaller proportion will experience events such as myocardial infarction or stroke. In order to predict CVD risk in the general population biomarkers have to be sufficiently sensitive to detect the increased risk but have to be specific in order to avoid large numbers of false positive results that could lead to unnecessary further investigations and potentially interventions.
- In disease cohorts, i.e. in people who already have CVD, the clinical requirements for biomarkers are based on prediction of events (e.g. myocardial infarction in a cohort of people

with stable coronary artery disease); prediction of disease progression and rate of disease progression; and monitoring of response to therapies. In this scenario biomarkers have to be very specific for the outcome of interest in order to provide precise data that will inform treatment decisions.

The EU-MASCARA Consortium had access to a number of general population and disease cohorts for biomarker studies. These cohorts were all utilised to answer specific questions. The Consortium, however, also decided to focus on a smaller number of "core" cohorts for extended assessment of multiple biomarkers in order to collect information on whether a multimarker approach can provide substantially more information than targeted single-marker studies.

As a general result, and without going into detail already in this section, the Consortium found that in disease cohorts a limited number of highly specific biomarkers can predict disease outcome with high precision. The pathophysiology and the clinical background are known in this scenario and biomarkers can be selected on this knowledge. We have for example shown a range of microRNAs to be outcome markers in patients with heart failure based on their pathophysiological role (regulation of key target genes). In low risk scenarios such as in general population studies multiple factors can contribute to the development of CVD over a longer period of time. In order to capture the large number of potential disease pathways a large number of biomarkers (multimarker strategy) has to be employed. The "omics" based biomarkers from proteomic and metabolomic approaches performed best in this context.

Objective 3. Integration of emerging biomarkers reflecting different aspects of pathophysiology with established biomarkers into a common predictive model

The Consortium was aware of the risk that specific tasks would be met but their results would not be comprehensively analysed and brought together. This risk was inherent to the objectives of the call where partners with specific expertise were brought together into a Consortium in order to contribute with their cohorts, technologies and knowledge to the project. We have therefore from the onset of the project put particular focus on two Work Packages that bring the scientific efforts of the Consortium together (see section 3.1.3). We established a strong clinical platform (WP1) to coordinate access to samples and clinical data and maintain an overview of the "core" projects and additional targeted projects that answered specific questions. We have also established WP7 as a platform at the other end of the data workflow. In this Work Package the Consortium brought together the data from experiments in WP2-6 and in particular the data from analysis of the "core" cohorts.

The integration of multiple biomarkers that was proposed in EU-MASCARA served several purposes:

- By bringing together multiple layers of data the most promising biomarkers can be selected, thus allowing researchers and clinicians in the future to focus on tests that provide the best value whilst avoiding biomarker tests that do not have a reasonable diagnostic or predictive potential.
- Integrating biomarkers that reflect different aspects of the disease process can produce a network of potentially dysregulated pathways on which individual patients can then be mapped based on their biomarker data. Such complex networks with complex biomarker mapping have the potential to provide precise information on disease state and inform therapeutic decisions accordingly.
- Data integration can also identify gaps in networks and predict additional biomarkers and disease processes that were not covered by the original biomarker studies. Such networks and nodes can be developed on the basis of data generated by the Consortium. They can,

however, also be generated based on available literature data. The EU-MASCARA Consortium has decided to use both a data-driven and a literature-based approach to develop disease networks that provide holistic views of the pathophysiology of CVD.

Apart from statistical and bioinformatics-based data integration another key aspect of the work covered the evaluation of health economic benefits of emerging biomarkers. We have again used a data-driven approach using urinary proteomics as a specific example and a literature-based approach that modelled novel therapies against novel biomarkers in order to find thresholds for the costs of both diagnostic and therapeutic approaches to be cost effective.

Objective 4. Development of novel diagnostic test strategies to improve clinical management of patients with cardiovascular diseases

The ultimate aim of the project was the development of novel test strategies for CVD. This objective is not simply the development of a specific commercially available assay, but in the first instance it means the provision of information on selection of the best biomarkers for a given context of use; optimisation of analytical platforms; assessment of stability of biomarkers and reproducibility of results; and methods to analyse and interpret data, especially when they derive from multidimensional "omics" experiments or other multimarker approaches.

As briefly mentioned under Objective 2 one of the crucial strategies is the choice of the right test for a given clinical scenario, e.g. for prediction of disease in the general population or prediction of events in patients who already have CVD. The Consortium has studied the appropriate test strategies extensively throughout the duration of the project.

With regard to commercialisation of any findings, the EU-MASCARA Consortium had a particular responsibility for the SME partners. It was important for the project to provide SME partners with data that help them to further develop their business by developing specific biomarker tests. Results on this important task are provided in section 4 of this document. We would like to emphasise here already that the objective has been met albeit at different stages of assay development for different SME partners. Whereas for example partner Mosaiques Diagnostics GmbH have already developed a commercially available urinary proteomics based assay the development of commercial products that specifically derive from EU-MASCARA foreground are less advanced for partners ACS Biomarker and Randox Testing Services. The former, however, were able to miniaturise their analytical procedures and to focus their future development on the most promising prognostic microRNAs; the latter have produced data on reproducibility of results and gained insight in concentration ranges of multiple biomarkers in people with CVD which will help them develop more specific biomarker arrays as part of their future strategies.

3. Description of the main S&T results/foregrounds

The EU-MASCARA Consortium has achieved its tasks and provided detailed delivery reports and periodic reports throughout the course of the project. We will now take the opportunity to highlight key achievements of the project. This report highlights the broad themes whilst providing sufficient detail of specific achievements.

The Consortium has decided to draft this report by following the original Work Package structure. Thereby the results can be seen in their original context, naturally culminating in WP7 with an integrative view on the data. In the following section, however, this report will first describe the key concepts and the infrastructure of the project; how they have been met throughout the course of the programme; and how they have developed and matured into a platform that will support collaborative research beyond the lifetime of EU-MASCARA.

3.1 Infrastructure of the EU-MASCARA project

3.1.1 Key concepts for the delivery of success

The EU-MASCARA project was based on eight key concepts that were unique to this Consortium and provided an important basis for delivery of the ambitious work programme.

Key concept 1: Existing collaboration between partners of EU-MASCARA

Partners of EU-MASCARA have collaborated already before the research proposal has been drafted and submitted to the European Commission. This existing collaboration facilitated a swift start of the project without the need to establish project specific communication channels. Partners were aware of skills and resources of other partners and continued direct collaboration beyond the immediate tasks of this project throughout and beyond the lifetime of EU-MASCARA. In fact, the existing collaborations were so close that regular meetings of partners happened naturally at conferences and workshops and beyond the annual Consortium meetings and few Consortium-wide teleconferences there was no need to steer the project in specific directions as all partners were fully committed to the objectives as part of their ongoing research activities.

Key concept 2: Availability of specimens for biomarker analysis.

The call text asked for available cohorts with available samples and not for establishment of new clinical cohorts. The Consortium fully subscribed to this concept as new clinical studies would have caused significant delays in delivering the key objectives of this project. A number of clinical cohorts with available bio samples have been proposed and throughout the project all of these samples were made available to the Consortium partners (Table 1).

We would, however, like to mention a few changes to the original concept that will explain some of the new directions that the Consortium has taken:

- Against the original assumption not all of the cohorts were readily available to the Consortium in their proposed form. This was mainly an issue of ownership where an investigator moved from one institution to another. In particular, the access to the Aldo-DHF cohort was delayed for this reason and the Consortium is grateful to the Commission for granting a 6-month extension to be able to capitalise on this important clinical cohort.
- Some of the cohorts saw further recruitment of study participants throughout the course of EU-MASCARA. It should be noted that such additional recruitment did not result in additional costs for project partners as these activities were funded from internal or other

sources. An exception was the follow-up of a few selected cohorts including the InGenious HyperCare cohort that were explicitly mentioned in the application. Overall the ongoing recruitment considerably increased the available sample sizes and numbers of events during follow-up.

- It was clear that the Consortium cannot measure all available biomarkers across all >30,000 samples from the available cohorts. Instead, the Consortium has defined "core" cohorts for comprehensive biomarker assessment (InGenious HyperCare, FLEMENGHO and Generation Scotland) and used the other cohorts and associated samples for specific questions related to specific biomarkers.
- Throughout the course of the EU-MASCARA project partners got access to additional cohorts that were not part of the original proposal. The Consortium decided to integrate such additional cohorts into the work programme if no further costs were generated; the additional cohorts provide information that is not sufficiently covered by existing cohorts; and access was granted to all Consortium partners as required. The Rio-Hortega study and the extended follow-up of FLEMENGHO are examples of such additional activities.
- The Consortium brought in additional cohorts where new developments that were not foreseen at the time of application more than five years ago required an adaptation of the work programme or opened new opportunities. For example, the notion that history of pre-eclampsia is an important cardiovascular risk factor and that the pathogenetic principles that lead to pre-eclampsia could also play a role in other CVDs led partner University of Glasgow bring in their cohorts of women with history of pre-eclampsia for biomarker studies.

Partner	Cohort	N	Type	Principal phenotype	Follow-up
AUX	a) InGenious HyperCare	2000	Families	Hypertension	4 years
	b) InGenious HyperCare	500	Families	Early stroke	3 years
	c) Piancavallo	500	Patients	Severe obesity	2 years
RWTH	NT^{CVD}	450	Patients	CVD in CKD	>3 years
INCLIVA	a) n/a	300	Patients	Hypertension	>5 years
	b) Valladolid	1500	Population	General population	>5 years
FIMA	a) CUN	1000	Patients	Hypertension	n/a
	b) RIVANA	900	Patients	Metabolic syndrome	10 years
	c) LEIZARAN	250	Patients	Heart failure	n/a
GLA	Generation Scotland	22,000	Population	Blood pressure	3 years
CHA	Aldo-DHF	422	Patients	Diastolic heart failure	1 year
LEU	FLEMENGHO	3,600	Families	Blood pressure	>15 years
MHH	ROADMAP	4,449	Patients	Type 2 diabetes	3 years
MIB	a) PAMELA	3,200	Population	Blood pressure	>15 years
	b) Monza	1,500	Patients	Hypertension	3 years
UMA	a) PRIMA	364	Patients	Heart failure	2 years
	b) Cardiomyopathy	150	Patients	Dilated cardiomyopathy	5 years
	c) TIME-CHF	499	Patients	Heart failure	5 years

Table 1. Patient and population cohorts available to EU-MASCARA for biomarker analysis.

Key concept 3: Availability of cohorts with high-fidelity phenotyping

Studies into markers for prediction and diagnosis of clinically relevant conditions (e.g. coronary artery disease or heart failure) and events (e.g. hospitalisation, myocardial infarction or death) were the key objectives of the programme. However, such diagnoses and events are rare in the general

population and prediction over a lifespan is technically and conceptually challenging. Therefore, additional "intermediate" phenotypes such as endothelial function, vascular stiffness and left ventricular mass are important surrogates for the assessment of cardiovascular health. Deterioration in these phenotypes over time can translate to increased cardiovascular risk. The Consortium has selected cohorts where high-fidelity phenotypes are available in order to answer specific biomarker related questions.

Key concept 4: Availability of cohorts covering the whole range of the cardiovascular continuum

The clinical cohorts covered the whole spectrum of disease from apparently healthy people in the general population to patients with advanced CVD such as heart failure at NYHA stage 3. The availability of a broad spectrum of conditions allowed the Consortium to develop and validate context-specific biomarkers. This is an important requirement especially for future commercialisation and translation into clinical practice.

Key concept 5: Availability of cohorts from different European regions

The population and disease cohorts brought into the Consortium covered a wide range of European regions particularly in the north, middle, west and south of Europe. Findings from the project are therefore translatable into other European regions. We acknowledge that Eastern Europe has been underrepresented in the composition of EU-MASCARA and its resources. In part this limitation has been addressed in programmes run by the partners in parallel to EU-MASCARA such as PRIORITY, iMODE-CKD and HOMAGE which also use biomarker approaches in CVD and have partners from Eastern Europe. In addition, in the InGenious HyperCare cohort patients from Poland and Czech Republic were present therefore there is representation with regards to the cohorts used. The FLEMENGHO data can be translated to cohorts within the EPOGH collaboration that employs similar clinical protocols across a wider coverage of Europe.

Key concept 6: Availability of patient and population cohorts

As mentioned above the context of use is important for biomarker applications. Availability of population samples (low disease prevalence, low risk of events) and specific patient cohorts (disease prevalence throughout, high risk of events) allowed the Consortium to analyse biomarkers across a spectrum of clinical scenarios.

Key concept 7: Evaluation and validation of robust and clinically useful biomarkers

The proposal was built upon available biomarkers that were excellent candidates for clinical use as cardiovascular risk markers. All biomarkers that were proposed in the application had sufficient backup from previous studies to justify their further evaluation and validation and ranged from single biomarkers such as albuminuria to complex multidimensional biomarkers including microRNA profiles, proteomic and metabolomic data.

The Consortium, in keeping with the call text, didn’t propose to develop new biomarker as part of the project work. However, during the course of action, several opportunities have arisen to use the existing infrastructure and analysis pipelines to quickly evaluate novel biomarkers that either appeared in the literature or were discovered by the partners. We had probably underestimated this potential and in the end we were grateful for the flexibility of the programme that allowed us to capitalise on new discoveries. Examples include the newly discovered vasoactive peptides that derived from plasma proteomic studies; circulating mitochondrial DNA as a novel marker of oxidative damage; and the description of microRNAs that are dysregulated in pre-eclampsia.

The opportunity to add value to the project work programme without compromising the delivery of the originally proposed tasks, was an important feature of this project over its entire funding period and is the foundation for future collaborative work between the partners.

Key concept 8: Strong integrative and translational platform

As mentioned above an ambitious work programme comes with the risk that the disjointed data produced will not be evaluated comprehensively. The Consortium has therefore developed a dedicated Work Package on data integration that feature prominently in the description of main results in section 3.2.7.

3.1.2 Timelines of the project

EU-MASCARA was designed as a 4-year collaborative project as outlined in Figure 1.

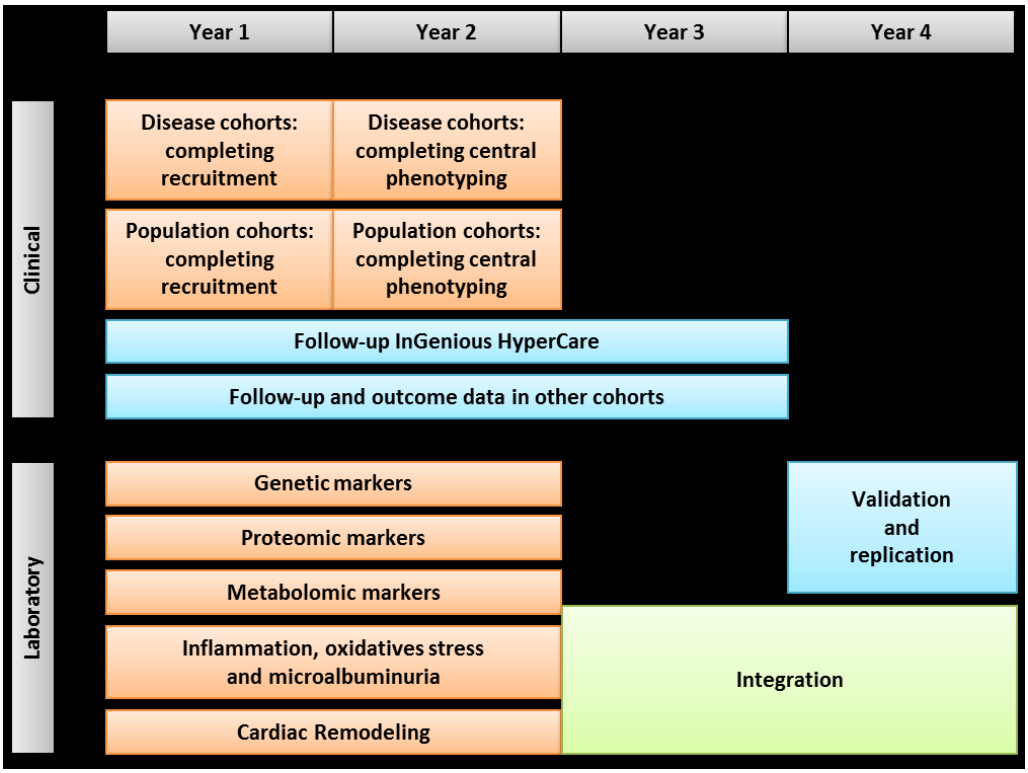

Figure 1. Originally proposed project timelines.

This original concept had a clear distinction between a first biomarker validation step in cross-sectional cohorts and a second step in longitudinal/prospective cohorts after integrative data analysis of biomarker data.

The EU-MASCARA Consortium broadly followed this track but added additional options as the project matured:

- A strict separation between cross-sectional and longitudinal cohorts was abandoned. This was for two reasons. First, some of the available cohorts provided high quality baseline and follow-up data that could be analysed for both features. A prime example is the FLEMENGHO cohort. Second, some of the data from longitudinal analysis benefited from

additional mechanistic work and the Consortium allowed partners to go back to such data in order to gain deeper insight in the pathophysiological processes.

- Recruitment of participants into clinical studies continued well beyond Year 1 and in some occasions until the end of the project. As outlined above this activity didn't resulted in additional costs and was done over and above the rich data that were available at the beginning of the project and at Year 1. However, some of the longitudinal analyses and also the future opportunities for collaboration beyond the project's lifetime will benefit from larger sample sizes.
- There has been some delay with logistic aspects of the project. Distribution of aliquots of sample across many partner laboratories was more challenging than originally anticipated and access to some cohort data and samples was delayed due to ownership issues. Therefore, the Consortium decided to apply for a 6-month extension of the project period in order to address three main aspects that otherwise wouldn't be delivered to their full potential: (1) Randox Testing Services received samples later than originally planned and was then able to provide a full set of data within the extended project lifetime; (2) the Aldo-DHF cohort was fully available for analysis only at the end of the regular funding period and with the extension analysis of microRNAs in this cohort could be achieved; (3) the Consortium was able to respond to recent developments in biomarker research and further improved the quality of data, e.g. by providing additional sequence data in proteomics experiments and by performing lipidomics studies.

3.1.3 Work Packages

The project was organised in seven research based Work Packages (WP1-7) and two Work Packages on project management and dissemination/exploitation (WP8 and WP9) (Figure 2). This document follows the Work Package structure to highlight key achievements of the project. Outputs from the individual project partners' perspective are presented in the impact section 4.

As mentioned above WPs 1 and 7 provided support for the whole project coordinating data input and output/analysis, respectively. Other Work Packages (WPs 2-6) had distinct tasks, many of which required input from WPs 1 and 7 and collaboration between individual partners. In fact, the Consortium decided at its third Consortium Meeting in Milan to encourage such bilateral and trilateral collaborations directly between partners within the context of the work programme as this was the most efficient way to generate data quickly and without unnecessary administrative burden.

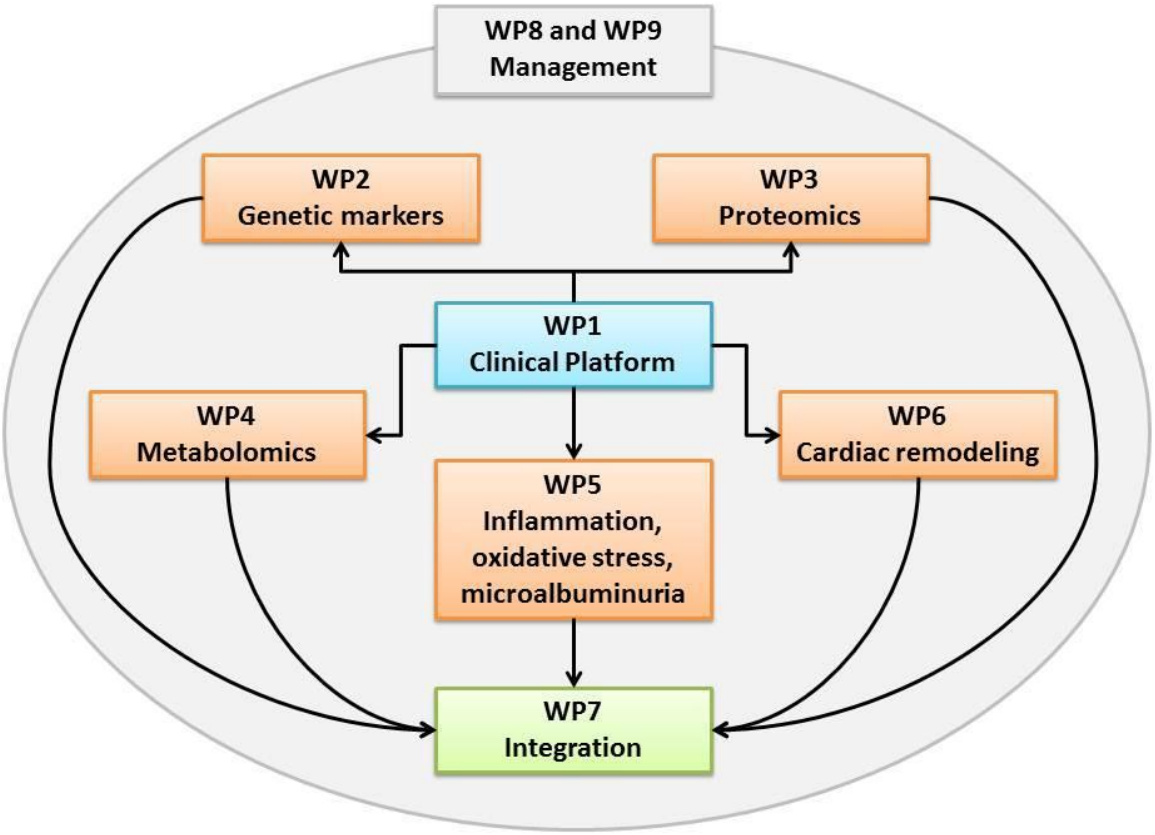

Figure 2. Work Packages of EU-MASCARA.

3.2 The EU-MASCARA work packages and major scientific results

3.2.1 Work package 1: Clinical platform

The clinical platform in WP1 coordinated access to all samples and clinical data. Clinical data included diagnosis of CVD, cardiovascular events and all intermediate phenotypes such as vascular stiffness and left ventricular mass. WP1 has been led by the team at **Istituto Auxologico**.

Key achievements

1. Data from the various cohorts or families of individuals with cardiovascular disease or at high cardiovascular risk available to Consortium partners have been entered into the project database, and further individuals have been recruited into several of these cohorts.
2. Phenotypes of subclinical organ damage have been standardised with central reading of echocardiograms recorded in families with a hypertensive member within the InGenious HyperCare family cohort.
3. Prospective follow-up has continued within a number of the EU-MASCARA cohorts, such as the InGenious HyperCare cohort, Generation Scotland – Scottish Family Health Study, FLEMENGHO, Monza, Hortega, Lapunte Study, Aldo-DHF Study, PRIMA-Study, Cardiomyopathy cohort, TIME-CHF, Multi-Marker Study, NTCVA, and CVOC cohort.
4. Biosamples from most of the clinical units of the InGenious HyperCare study have been centralized and stocked at AUX, and have been made available to the EU-MASCARA partners.

Within the framework of WP1 the Consortium also studied the role of non-circulating biomarkers (e.g. imaging studies or functional properties of blood vessels and the heart) in the disease process and as predictors of cardiovascular events. A large number of such studies has been conducted for two reasons. First, in-depth phenotypic data were collected as part of WP1 activities or were already available in the studies that were brought into EU-MASCARA. The tasks of WP1 were related to collection and description of these study cohorts with hypertension, heart failure, renal disease, stroke and other cardiovascular conditions as well as of the general population cohorts. Second, many of these phenotypic markers are important predictors of events and any novel markers have to compete with them in order to demonstrate added or alternative value.

In this report we will highlight some of the specific results that derived from WP1.

The team at the University of Glasgow has conducted a study to assess **vascular function and structure in women who had pre-eclampsia** during a pregnancy up to 30 years ago. It is known that these women are at increased cardiovascular risk but the mechanisms are poorly understood. A number of vascular markers including endothelial function, vascular stiffness and carotid intima-media thickness were assessed in 86 cases and 80 controls. The research team found impaired function of the vascular endothelium (assessed by flow-mediated dilatation) in women with a history of pre-eclampsia that was independent of other risk factors including blood pressure and age. Other markers such as pulse wave velocity were also different between the groups but not after adjustment for other cardiovascular risk factors (Figure 3).

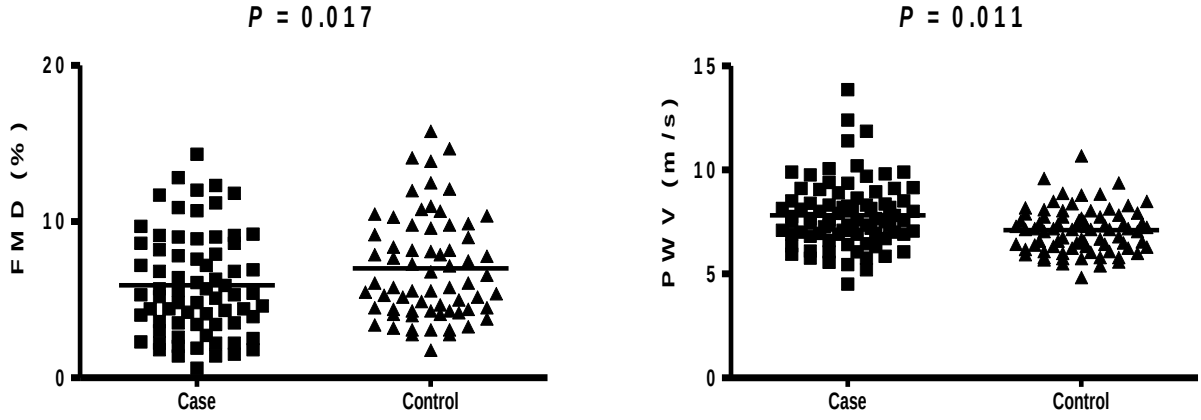

Figure 3. Left panel. There was a significant difference in endothelial function (flow mediated dilation; FMD) between women who had pre-eclampsia (cases) and controls that remained significant after adjustment for age, body mass index and systolic blood pressure. Right panel. Women with history of pre-eclampsia also had greater vascular stiffness (pulse wave velocity; PWV) but this was not statistically significant after adjustment for other risk factors. Unadjusted P-values are displayed in the graphs.

An important finding is that in these women, who are apparently healthy and who are, apart from their history of pre-eclampsia many years ago, not different in their risk factor profiles from controls, we found evidence of early changes to vascular function. The cohort was then also used for further biomarker studies within WPs 3, 4 and 5.

The Team at K.U. Leuven validated a method to study the **diameter of retinal microvessels**. These blood vessels are closely linked to the cerebral vasculature and offer an opportunity to study the properties of small vessels in humans without any invasive procedures. Their study is important for investigating the mechanism of stroke but also of other CVDs. The diameter of vessels changes slightly with the heart beat when they fill with blood but whether this effect that is clearly present in the large arteries close to the heart also affects the very small vessels in the eye was not clear. Traditionally the measurements are gated to an ECG reading, to make sure that they will be taken

during the same phase of the cardiac cycle. The team in Leuven have shown, however, that such ECG gating does not improve the quality of the readings. These data will allow researchers in the future to use archived retina photographs for the study of microvessels and how their diameter predicts cardiovascular outcome.

The team at the University of Maastricht performed biomarker studies in patients with heart failure and especially in patients with dilated cardiomyopathy (DCM). These patients undergo detailed clinical investigations and in suspected DCM this involves a cardiac biopsy. As part of their studies to characterise patients with DCM within the EU-MASCARA project the Maastricht team have looked at the **prognostic value of cardiac arrhythmias** at the time of biopsy for future cardiovascular events. They found that irrespective of the type (duration/frequency) patients with arrhythmias have poorer prognosis than those without arrhythmias.

3.2.2 Work package 2: Genetic markers of cardiovascular disease

WP2 coordinated studies into genetic markers of CVD. In keeping with the call text the EU-MASCARA Consortium has not performed new genetic discovery studies such as genome-wide association or gene sequencing studies but focussed on plausible candidate genes that could be used as risk markers based on their role in pathophysiology of CVD. WP2 has been led by the team at **K.U. Leuven**.

Key achievements

1. Prospective follow-up of the FLEMENGHO cohort with repeat high-fidelity vascular and echocardiographic phenotyping to support prognostic studies into genetic markers of CVD.
2. Establishment of significant heritability of diastolic left ventricular function in the combined family-based FLEMENGHO and EPOGH cohort.
3. Association of diastolic LV function with genetic variation in *ATP12A* and *PRK61* and association of the *AGTR1* A1166C polymorphism (a binding site of miRNA-155) with LV mass.
4. Demonstration in the FLEMENGHO cohort that rs3918226 in *NOS3* is a hypertension susceptibility gene.
5. Demonstration that in pre-eclampsia miRNA-206 is upregulated in the maternal circulation and placental tissue and that expression of genes encoding HLA-A and HLA-DRB1 are upregulated in the placenta.
6. Association of various types of cardiomyopathy with specific miRNA-profiles.
7. ACS Biomarker validated a series 14 of miRNAs for heart failure.
8. In heart failure patients *NPPB* T-381C influences the high end of NT-proBNP levels. NT-proBNP-guided therapy reduced the frequency of high-end levels in CC-carriers.

In this report we will highlight some of the specific results that derived from WP2.

The team at the University of Glasgow studied **genes that are involved in Long QT Syndrome** (LQTS). LQTS predisposes affected patients to potentially life threatening arrhythmias. Details of the genetic make-up of patients with LQTS and if mutations in key genes associated with the conditions are prevalent in the general population required further studies that were conducted in Glasgow. In the Scottish population there was an association between the rs12143842 variant in the *NOS1AP* gene and QTc duration. It has also been found that prolonged QTc interval in one parent is associated with increased risk of QT prolongation (odds ratio 2.44) in the offspring.

The team at K.U. Leuven translated functional data that derived from experiments into platelet aggregation into cardiovascular risk prediction. **Platelet Endothelial Aggregation Receptor 1** (PEAR1) is a membrane protein highly expressed in platelets and endothelial cells. PEAR1 mediates

platelet contact-induced activation and sustained platelet aggregation. The association of variants in the *PEAR1* gene and cardiovascular outcome was studied in 1938 participants randomly recruited from a Flemish population. The investigators could not replicate previous reports suggesting that *PEAR1* might be a susceptibility gene for cardiovascular complications. Such apparently "negative" data are important as they help to rule out possible candidate biomarkers (genetic variants in this case) from further development as risk predictors.

In a collaboration between ACS Biomarker and Charité Universitätsmedizin Berlin the Aldo-DHF cohort was used to study the potential use of **microRNA profiles** for prediction of cardiovascular events in patients with diastolic heart failure. A large number of microRNAs were studied and compared with established cardiovascular risk factors and NT-proBNP levels to assess their added prognostic value.

Further analysis in this cohort demonstrated that five microRNAs were associated with changes in NT-proBNP over time, i.e. with improvement or worsening of heart failure.

As part of WP2 (but also as part of other Work Packages) the Consortium originally proposed to develop a novel bioassay that would assess relevant biomarkers for cardiovascular risk prediction simultaneously. It became clear during the course of the project that with the current technologies, an assay measuring a wide variety of different analytes, such as miRNAs, proteins and metabolites and thereby provide a full biomarker profile is impossible. The Consortium has therefore decided not to pursue this task and instead focus on analysis of biomarker data that in the future can help to shape such developments.

3.2.3 Work package 3: Proteomic markers of cardiovascular disease

WP3 provided a platform for studies into proteomic markers of CVD. Large-scale protein and peptide expression data were generated from blood and urine samples and their association with CVD and intermediate phenotypes was assessed in a first stage. In particular, biomarkers that were generated from urine samples were found to be promising candidates as predictive markers and were evaluated longitudinally. WP3 has been led by the team at **Mosaiques Diagnostics GmbH**.

Key achievements

1. New proteomic urinary peptide biomarker patterns specific for heart failure with reduced ejection fraction (HFrEF), and the prediction of acute coronary syndromes (ACS; myocardial infarction and unstable angina pectoris) due to rupture of unstable atherosclerotic plaques as well as of the progression from asymptomatic left ventricular dysfunction to heart failure have been identified (Figure 5).
2. Based on these biomarker patterns, diagnostic (HFREF103 for HFrEF) and prognostic (ACSPC for ACS, LVHFP for progression to heart failure) disease/event classifiers have been established in order to identify individuals with HFrEF/LVHF or at risk for ACS and heart failure. Already established classifiers, based on proteomic urinary peptide biomarker patterns specific for diastolic left ventricular dysfunction (DLVD) and coronary artery disease (CAD), have been further validated in the EPOGH, InGenious HyperCare and ACSOT cohorts. Thereby the CAD classifier revealed some predictive potential for CAD endpoints like non-fatal myocardial infarction. The diagnostic and predictive power of the DLVD classifier has been confirmed.
3. Sequencing of proteomic biomarkers commenced almost 1 year ahead of schedule as MOS already had access to >1000 datasets from experiments that were brought into the EU-MASCARA project. Oxidative modifications of the amino acids proline and methionine have

been found. Sequencing of peptides of various disease/event-specific peptide biomarker patterns will continue beyond the lifetime of the project.

4. A "multivariable predictor model" based on plasma proteomic data from the InGenious HyperCare cohort has been developed that characterises patients with hypertension.

In this report we will highlight some of the specific results that derived from WP3.

The team in Glasgow has led a study to further validate a previously established urinary proteomics-based composite **biomarker for coronary artery disease** (CAD238). In a cross-sectional study an association between severity of CAD (assessed by the Gensini score) and CAD238 classifier values was found that provides further evidence for a direct reflection of pathophysiological processes and cardiovascular phenotypes by this biomarker panel (Figure 4).

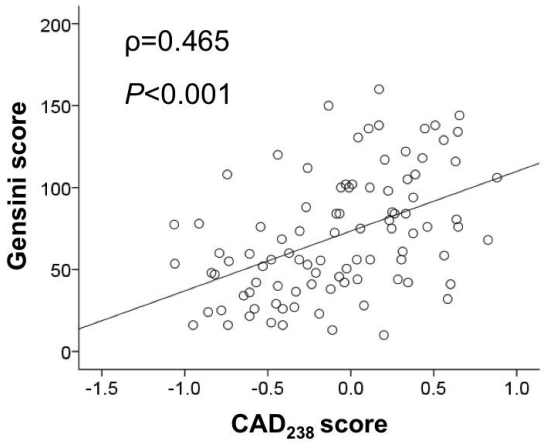

Figure 4. Correlation between CAD238 score and the Gensini score. The Gensini score (y-axis) is plotted against the CAD238 score (x-axis) for 96 patients. Shown are the Spearman's correlation coefficient and the corresponding *P*-value.

The team at RWTH identified 14 molecular features in the NTCVD study by ESI-LC/MS-MS that classify the status of the patients with **chronic kidney disease** and identified Sodium bicarbonate transporter like proteinc collagen fragment, erythrozyte membran glycopeptide, osteocalcin, thymosin beta-10, humanin, aldehydrogenase family 1, sodium bicarbonate transporter like protein and amilo-sensitive-amino-oxidase as the underlying substances (Figure 5).

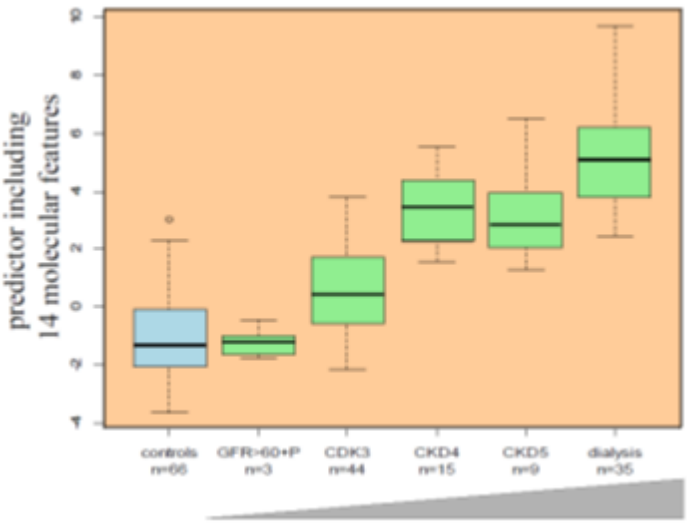

Figure 5. Stages of chronic kidney disease (CKD) and levels of a 14-molecule plasma proteomic classifier derived from ESI-LC/MS-MS experiments.

3.2.4 Work package 4: Metabolomic markers of cardiovascular disease

WP4 coordinated metabolomic studies in samples from patients with CVD. The experiments were performed by the team at **INCLIVA** who also led the Work Package. Samples from the EU-MASCARA "core" cohorts were analysed as well as samples from other cohorts including the Rio-Hortega cohort.

Key achievements

1. Platform robust and stable for metabolomics analysis. More than 5,000 samples measured in the context of the project.
2. Detection of genotypes associated to predisposition and resistance to microalbuminuria by stratified metabolomics-genetics analysis of the Rio Hortega Valledolid cohort.
3. Detection of metabolomics markers of pre-eclampsia. 4) Detection of metabolomics signature of diastolic left ventricular function in the FLEMENGHO cohort. 5) Detection of genomic and metabolomic profile associated to clustering of cardio-metabolic risk factors in the Rio Hortega Valledolid cohort.

In this report we will highlight some of the specific results that derived from WP4.

The team at INCLIVA analysed **metabolomic profiles in patients with and without microalbuminuria**. Microalbuminuria is a universal cardiovascular risk marker that broadly reflects the status of the systemic and renal microvasculature. As with other CVD entities it remains unclear why not all the patients with similar risk factor profiles develop organ damage. The study of markers associated with microalbuminuria will provide insights into the pathophysiology of the condition. Metabolomic analysis was performed using the Valencia Metabolomics platform. A principal component analysis (PCA) for serum was firstly performed corresponding to an unsupervised multivariate data reduction routine, which serves to rapidly evaluate the data distribution and inter-sample similarities (e.g., clusterings and outliers). After PCA analysis, a partial least-squares discriminant analysis (PLS-DA) was used to build a statistical model that optimizes the separation between the two groups (subjects with and without microalbuminuria (Figure 6).

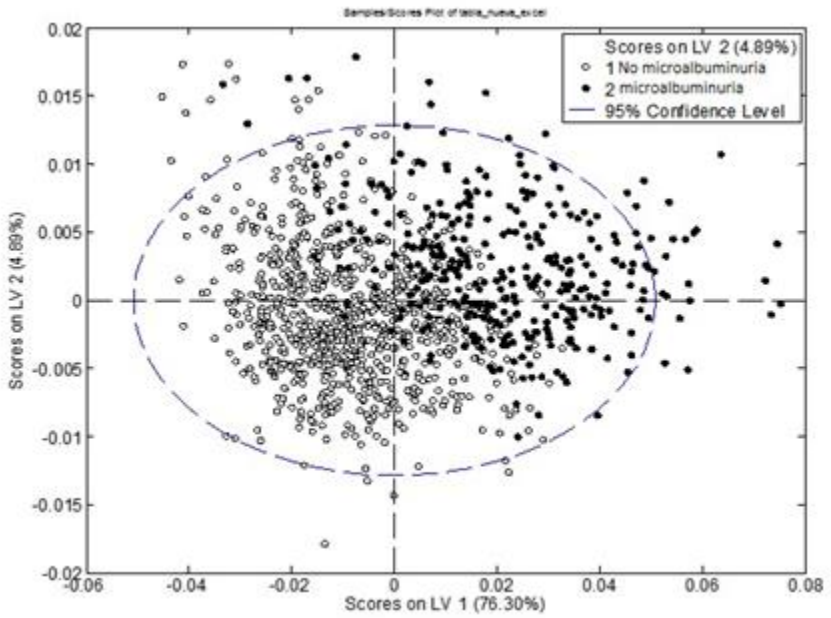

Figure 6. PLS-DA model scores plot for discrimination between patients without (open circles) and with microalbuminuria (close circles) based on the NMR spectra of blood serum of the entire cohort.

The differentially regulated endogenous compounds detected, include mitochondrial metabolism (citrate), extra mitochondrial metabolism (glucose, pyruvate, lactate, creatinine, creatine, creatine phosphate) and several amino acids and their derivative signals (such as proline, glutamine, N-acetylglutamine, alanine). Among these, branched amino acids (valine, isoleucine, leucine, 3-hydroxyisovalerate) exhibited a relatively high statistical significance. We also detected numerous fatty acid signals, (FA-CH3, FA-CH2-CH2CO, FA-CH2-CH3), as well as signals from cholesterol, choline and phosphocholine, aminobutyrate, dimetylamine, trimethylamine, and albumin.

The team at RWTH was also involved in the analysis of albumin by studying **post-translational modifications of albumin**. Albumin isolated from plasma of patients with CVD but not from healthy control subjects, was found to be specifically post-translationally modified by guanidylation of lysines at positions 249, 468, 548, 565 and 588. After identification of guanidylations as post-translational modifications of albumin in CVD, *in vitro* experiments into post-translational guanidylation of albumin were performed. A direct effect on the binding capacity of hydrophobic metabolites like indoxyl sulfate and tryptophan has been demonstrated, which links this result to metabolomic studies performed at INCLIVA within WP4.

With regard to the **association of metabolomic markers with cardiovascular phenotypes such as diastolic left ventricular function** the team at K.U. Leuven led the collaborative study of 711 apparently healthy subjects. Using the PLS-DA approach (see above), metabolites inversely associated with diastolic left ventricular dysfunction included glucose + glutamine, glucose + 2 aminobutyrate, and glucose + 2 phosphoglycerate. These combined, increased (P<0.0001) diagnostic accuracy over and beyond NT-proBNP.

WP4 overall produced a rich set of data that will be further evaluated beyond the lifetime of the project. With baseline metabolomic profiles measured in a number of large cohorts within EU-MASCARA, there will be future collaborations to study pathophysiological aspects and predictive value related to these markers. This also refers to the additional lipidomics data that have been generated as part of the project extension and that will provide deeper insight into lipid metabolism compared to the routine NMR metabolomic data.

3.2.5 Work package 5: Inflammation, oxidative stress and microalbuminuria as biomarkers of cardiovascular disease

WP5 provided a platform for the analysis of specific markers of inflammation, oxidative stress and microalbuminuria in the context of CVD. This WP is a prime example of the close links between partners and Work Packages. Many of the markers assessed in WP5 were afterwards brought together with cardiovascular phenotypes (WP1) and the multidimensional omics based markers in WP3 and WP4. An example has already been provided in section 3.2.4 where the relationship between metabolomic markers and microalbuminuria has been described. WP5 has been led by the team at **Randox Testing Services**.

Key achievements

1. A total of 1903 serum samples from the FLEMENGHO cohort (K.U. Leuven) and the Generation Scotland – Scottish Family Health Study (University of Glasgow) have been measured by RTS using a number of Multiplex Protein Arrays.
2. The biomarker data were compared directly with specific cardiovascular phenotypes and were used in integrative analysis (WP7) together with other biomarkers, to describe specific phenotypes and cardiovascular outcomes.
3. An extensive range of inflammatory and oxidative stress related biomarkers have been analysed in the ROADMAP cohort to study their association with development of microalbuminuria in patients with type 2 diabetes.
4. Extensive quality controls have been performed in samples from patients with CVD and controls in order to better understand the stability of biomarkers in stored samples and reproducibility of results in repeat measurements.

Here are some more specific results that derived from WP5.

The team at Medizinische Hochschule Hannover studied the **association between markers of angiogenesis and inflammation and microalbuminuria** in the ROADMAP and OFU cohorts. Data in specific biomarkers are provided in Figure 7. The data show that the molecular phenotypes "microalbuminuria", "angiogenesis" and "inflammation" are tightly interlinked, explaining the universal value of microalbuminuria as an integrative biomarker of cardiovascular risk.

Associations between **cytokine profiles and left ventricular remodelling and dysfunction** have been analysed by the team at K.U. Leuven. Cytokines were measured using a 63-plex Luminex platform. Using partial least squares-discriminant analysis, we constructed three latent variables from the measured cytokines that explained 35%–45% of the variance between groups. We identified five common cytokines (interleukin 18, monokine induced by gamma interferon, hepatocyte growth factor, epithelial neutrophil-activating peptide 78, and vascular endothelial growth factor D) with a stable signal which had a major impact on the construction of the latent variables. Among these cytokines, after adjustment for confounders, interleukin 18 remained significantly different between hypertensive participants with and without left ventricular involvement ($P = 0.02$).

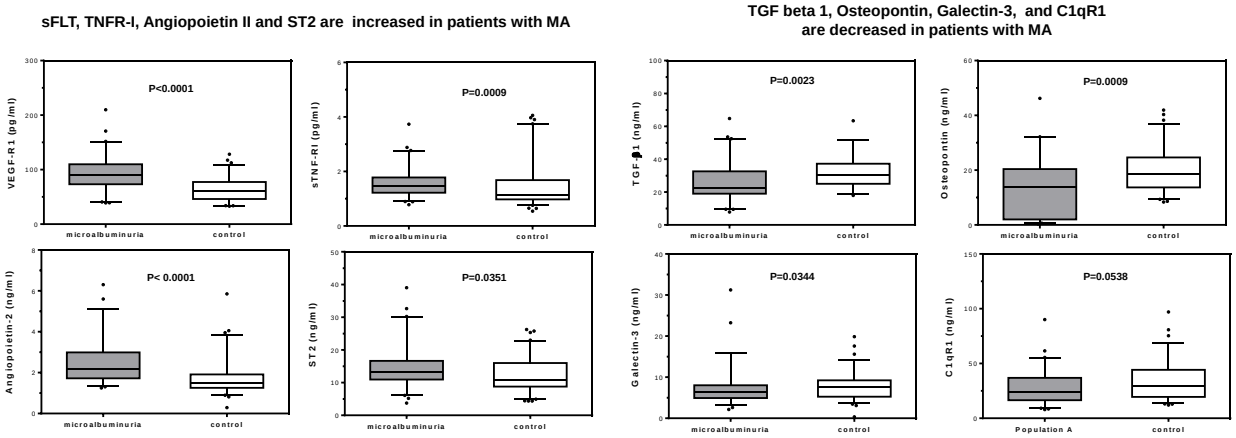

Figure 7. Differentially regulated protein biomarkers in patients with and without microalbuminuria. No significant differences were found for S100A8, endostatin, VAP-1, CXCL12, CXCL16, sTNFR-II, thrombomodulin, VEGF-A, copeptin, RAGE and angiopoietin-1.

The team at Randox Testing Services evaluated the **effect of freeze-thaw cycles on biomarker levels** systematically. Such studies are expensive and involve precious human samples, therefore they can only be conducted within consortia such as EU-MASCARA. For the Mets I array, RTS could only state evidence of trends - the study did not show a definitive increase/decrease in concentration after 1 freeze-thaw cycle. There seemed to be a large degree of inherent uncertainty of the measurement. Variation between the duplicates was ~5%, 7%, 7%, 10%, 12% & 60% for TNFa, Resistin, PAI, Leptin, IL6 & IL1a respectively. The high variation for the IL1a analyte was due to the results being below the assay sensitivity. For the Cytokine High Sensitivity Array there was also only evidence of trends. Again, there was quite a large degree of inherent uncertainty of measurement for several analytes. Variation between the duplicates is ~23% (IL2), 12% (IL4), 7% (IL6), 7% (IL8), 15% (IL10), 17% (IL1a), 18% (IL1b), 4% (EGF), 4% (MCP), 9% (TNFa), 6% (VEGF) & 49% (IFNg). The high variation of IFNg was due to the results being around the analytical sensitivity. These data are important for the future development of biochip arrays of cardiovascular markers and inform power calculations and data interpretation related to biomarker studies.

3.2.6 Work package 6: Biomarkers of myocardial remodelling

WP6 coordinated the analysis of biomarkers of cardiac remodelling. Markers of collagen turnover were of particular interest to the Consortium as they also provide information beyond the heart and may be a window into blood vessels and other organs that experience fibrotic changes as part of the pathophysiology of CVD. These markers also link to collagen fragments that have been assessed in WP3 using urinary proteomics by CE-MS. WP3 was coordinated by the team at **FIMA**.

Key achievements

1. The circulating biomarkers hs-cTnT and NT-proBNP have been confirmed as useful tools to detect subclinical left ventricular and left atrial remodelling in the population.
2. A set of urinary polypeptides has been proposed as a diagnostic tool to detect LV diastolic dysfunction in hypertensive patients, with subsequent confirmation in the general population, in which the prognostic value of this urinary classifier has also been demonstrated.
3. Findings in patients with heart failure confirm that circulating NT-proBNP and hs-cTnT may be considered as promising markers of discrimination and risk stratification, along with ST2 and cystatin C.

4. An association between cystatin C and alterations in collagen metabolism and left ventricular diastolic dysfunction has been demonstrated in patients with heart failure of hypertensive origin.
5. A new biomarker of myocardial collagen quality (i.e. collagen cross-linking) has been identified in patients with heart failure of hypertensive origin, with prognostic value to predict hospitalisation for heart failure.

In this report we will highlight some of the specific results that derived from WP6.

A **new circulating peptide with vasoregulatory activity** has been identified by the team at RWTH and has been assessed in patients with heart failure. The peptide with vasodilatory properties was chromatographically isolated from adrenal glands. The effects of this peptide were evaluated *in vitro* and *in vivo* and the receptor affinity was analysed. The sequence of the peptide isolated from human plasma was HSGFEDELSEVLENQSSQAELKEAVEEPSSKDVME. The vasoregulatory effects of this peptide is mediated by the AT2-receptor. This peptide impairs Ang-II-induced phosphorylation of the p38MAPK-pathway but not of ERK1/2. The plasma concentration was significantly increased in heart failure patients (Figure 8).

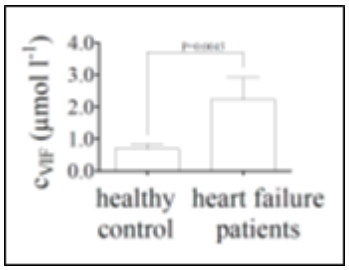

Figure 8. Plasma peptide concentration in patients with heart failure patients and controls with normal cardiac function (* p<0.05).

Together with the teams at Charité Universitätsmedizin Berlin and University of Glasgow, FIMA further confirmed the prognostic value of biomarkers of myocardial fibrosis and their ability to **monitor anti-fibrotic therapies** in the Aldo-DHF cohorts. 400 patients with heart failure with preserved ejection fraction were treated for 12 months with placebo or spironolactone, an aldosterone antagonist. The treatment with spironolactone resulted, beside other benefits, in decrease of diastolic function (E/e'). The carboxy-terminal propeptide of procollagen type I (PICP) has been used as a circulating biomarker of collagen type I synthesis.
In the Aldo-DHF cohort a clear difference in the amount of PICP was detectable between the placebo treated group and patients treated with spironolactone.

In collaboration between the teams in Glasgow and FIMA **markers of cardiac damage** have been assessed in the Generation Scotland – Scottish Family Health Study. This was an important effort to confirm the correct selection of patient groups out of more than 20,000 participants in the study. The results in Table 2 demonstrate the expected range of biomarker levels across patient groups.

	Controls (n=342)	Cardiac Disease (n=36)	Heart Failure (n=102)
Biomarkers of myocardial remodelling			
hs-cTnT, ng/l	7.3 (4.9 to 17.2)	8.5 (4.1 to 24.4)	**12.2 (6.1 to 32.7)***
NT-proBNP, pg/l	87.0 (30.1 to 338)	147 (14.5 to 586)	**293 (57.5 to 1909)*†**

Values are mean (±SD), geometric mean (10% to 90% interval) or number of subjects (%)

*P<0.05 vs controls †P<0.05 vs cardiac disease

Table 2. Markers of cardiac damage in the Generation Scotland – Scottish Family Health Study. Significant differences to controls were observed, as expected, for patients with heart failure.

As part of WP7 but mainly based on data from WP6 and therefore presented here, the Consortium performed integrative analyses in the InGenious HyperCare cohort in search for **biomarkers of left ventricular hypertrophy**. Seventeen clinical variables and 1605 molecular variables of various classes of biomolecules (microRNAs, peptides, proteins, metabolites) from blood and urine samples were analysed using simple and multiple linear regression. The first analytical approach involved a screening phase to detect candidate predictors, and a modelling phase using all statistically significant results as putative predictors. The second approach relied on principal components' analysis to reduce the dimensionality of the dataset. The first approach showed that a similar amount of data is explained by our clinical model as in the molecular model (adjusted R^2 = 0.209; 0.203). However, a model using both sets of data was more effective (adjusted R^2 = 0.333). The final 'combined' model consisted of four clinical variables (systolic blood pressure, heart rate, sex, history of congestive heart failure) and seven molecular variables (CO1A2, EFNA1, HCN4, PTGDS, phenylacetylglycine, an unidentifiable metabolite and a generalised measure of lipids), each with $P < 0.05$. Relevant biomarkers from regression analysis and adjustment for clinical features are shown in Table 3.

Molecule	Dataset (size)	Simple Linear Regression			With Confounders (Age, Sex, BMI)			With Clinical Model (Sex, SBP, HR, BMI)		
		Std. Beta	p_{β}	Adj. R^2	Std. Beta	p_{β}	Adj. R^2	Std. Beta	p_{β}	Adj. R^2
Angiotensin II	Identified Plasma Peptides (3)	0,18	1,00E-01	0,01	0,28	5,91E-04	0,14	0,22	4,89E-03	0,25
Angiotensin A	Identified Plasma Peptides (3)	0,17	1,00E-01	0,01	0,24	5,91E-04	0,15	0,19	4,89E-03	0,25
Unknown Peptide 266.16m/z	Unidentified Plasma Peptides (18)	0,43	1,05E-03	0,17	0,29	9,36E-02	0,23	0,33	1,03E-02	0,30
Unknown Peptide 288.13m/z	Unidentified Plasma Peptides (18)	0,30	4,18E-02	0,05	0,10	9,38E-01	0,17	0,15	5,77E-01	0,28
Unknown Peptide 567.14m/z	Unidentified Plasma Peptides (18)	0,37	4,18E-02	0,16	0,16	6,79E-01	0,26	0,13	7,29E-01	0,39
Unknown Peptide 834.18m/z	Unidentified Plasma Peptides (18)	0,32	4,18E-02	0,11	0,16	8,17E-01	0,29	0,27	1,68E-01	0,38
Unknown Peptide 831.18m/z	Unidentified Plasma Peptides (18)	0,31	4,18E-02	0,20	0,21	6,79E-01	0,31	0,09	8,51E-01	0,19
Unknown Peptide 553.10m/z	Unidentified Plasma Peptides (18)	0,32	3,06E-02	0,08	0,16	6,79E-01	0,08	0,21	3,00E-01	0,21
hsa-miR-18a-3p	Circulating miRNA (5)	0,23	4,30E-03	0,05	0,06	9,76E-01	0,12	0,01	9,50E-01	0,21
hsa-miR-92b-3p	Circulating miRNA (5)	0,18	2,60E-02	0,03	0,03	9,76E-01	0,11	0,00	9,50E-01	0,20
PICP/CITP (collagen accumulation)	Myocardial Remodelling Markers (4)	0,14	7,72E-02	0,01	0,17	1,62E-02	0,21	0,13	5,47E-02	0,31
Procollagen Type I (PICP)	Myocardial Remodelling Markers (4)	0,13	7,72E-02	0,01	0,14	3,41E-02	0,20	0,11	8,34E-02	0,32
isoleucine + L-proline + unsat f.a.	Serum Metabolites (50)	-0,27	1,31E-02	0,06	-0,14	2,74E-01	0,14	-0,15	5,40E-01	0,26
D-Glucose	Serum Metabolites (50)	0,24	1,71E-02	0,04	0,11	4,22E-01	0,13	0,10	5,40E-01	0,22
D-Glucose + L/D-Proline	Serum Metabolites (50)	0,27	1,71E-02	0,04	0,15	3,82E-01	0,15	0,12	5,40E-01	0,25
D-Glucose + alanine + glutamine	Serum Metabolites (50)	0,27	1,42E-02	0,05	0,10	4,82E-01	0,14	0,07	5,70E-01	0,23
Trimethylamine	Serum Metabolites (50)	-0,24	1,71E-02	0,05	-0,06	5,50E-01	0,14	-0,08	5,40E-01	0,23
'Unknown8'	Urinary Metabolites (168)	-0,35	9,49E-03	0,07	-0,19	4,94E-01	0,17	-0,14	3,40E-01	0,27
Phenylacetylglycine	Urinary Metabolites (168)	0,47	8,20E-02	0,03	0,50	2,15E-02	0,19	0,46	1,99E-02	0,29
Collagen alpha-1(I) chain	Urinary Peptides (1340)	-0,28	2,26E-02	0,06	-0,18	5,24E-01	0,17	-0,17	1,33E-01	0,27
Collagen alpha-1(III) chain	Urinary Peptides (1340)	-0,28	2,26E-02	0,06	-0,24	2,17E-02	0,21	-0,17	1,33E-01	0,30
Hemoglobin subunit beta	Urinary Peptides (1340)	-0,30	2,75E-02	0,06	-0,24	1,41E-01	0,22	-0,22	1,33E-01	0,31
Collagen alpha-1(I) chain	Urinary Peptides (1340)	-0,32	3,07E-02	0,05	-0,17	5,24E-01	0,20	-0,12	4,93E-01	0,31
Collagen alpha-1(I) chain	Urinary Peptides (1340)	-0,23	3,75E-02	0,05	-0,14	5,24E-01	0,18	-0,13	3,55E-01	0,31

Table 3. Molecular features associated with left ventricular mass derived from experiments into proteomics, metabolomics, inflammatory markers and markers of cardiac remodeling. Raw data, data adjusted for confounders and data integrated into a clinical model are displayed.

3.2.7 Work package 7: Integrative & systems' medicine based biomarkers of CVD: strategies for personalised medicine

WP7 played an essential role for the integration of data from WPs 1-6. The tasks were manifold and extended beyond a summary analysis of multiple biomarkers in specific cohorts but also involved the coordination of all projects that produced biomarker data. WP7 therefore worked in concert with WP1 (data input/data output). Beyond data analysis the health economic aspects of biomarker studies were analysed in WP7 and are presented here. WP7 was coordinated by the team at the **University of Glasgow**.

Key achievements

1. Cohorts with multiple sets of biomarkers were available to the Consortium and were analysed comprehensively. This included analysis of left ventricular mass in the InGenious HyperCare cohort, analysis of diagnosis and prediction of heart failure in the Generation Scotland – Scottish Family Health Study and the integrative analysis of circulating markers of collagen turnover and urinary collagens assessed by CE-MS.
2. Extensive literature mining has been performed to identify biomarkers that are associated with heart failure and that can be mapped to data from the EU-MASCARA Consortium.
3. Health economic analyses of the value of biomarker assessment to inform preventative treatment decisions in patients at different stages of cardiovascular risk have been performed.

In this report we will highlight some of the specific results that derived from WP7.

The team at Emergentec Biodevelopment GmbH led the **analysis of a molecular model that was based on published data in hypertension and heart failure**. Out of 107,464 publications with main focus on human hypertension (identified with the PubMed query "hypertension[majr:noexp] AND humans[mh]") a set of 1,202 protein coding genes could be extracted which formed the basis for generating the hypertension molecular model. Following the model forming procedure using the omicsNET framework as underlying biological network, a molecular model holding 20 process units and 369 proteins was derived. In order to generate a list of heart failure protein biomarkers with literature evidence the following query was used to search for publications in PubMed: heart failure[majr:noexp] AND biological markers[mh:noexp] AND humans[mh] NOT heart failure/genetics[majr:noexp]. 109 proteins could be extracted out of the set of 2,743 publications via gene2pubmed mapping. 20 of these markers were part of the hypertension molecular model with the assignment to the individual process units given in Figure 9.

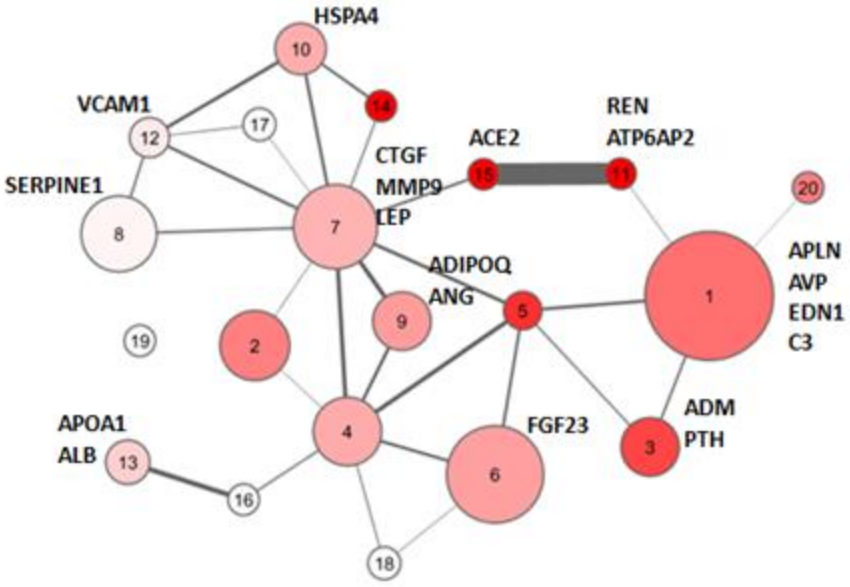

Figure 9. Heart failure biomarkers assigned to the Hypertension molecular model process units. Check with Paul that this is the correct version!

All proteins being part of the hypertension molecular model which are measurable by one of the EU-MASCARA partners either directly or via an associated miRNA or metabolite were then selected. The assignment to the respective hypertension process unit is given as well as which platform

technology can be used for measuring the respective molecule. Out of these, the following protein coding genes have been included in the Ingenious HyperCare cohort, either directly on the protein level or via a linked metabolite or peptide fragment: C3 (urinary proteomics), ANG (serum metabolomics), MMP9 (RTS assay), ALB (urinary proteomics), APOA1 (urinary proteomics). Based on concentration levels of analytes addressing these five biomarkers the set of patients in the Ingenious HyperCare cohort was segmented as given in Figure 10. Thirteen clusters could be identified ranging in size from 1 to 67 patients.

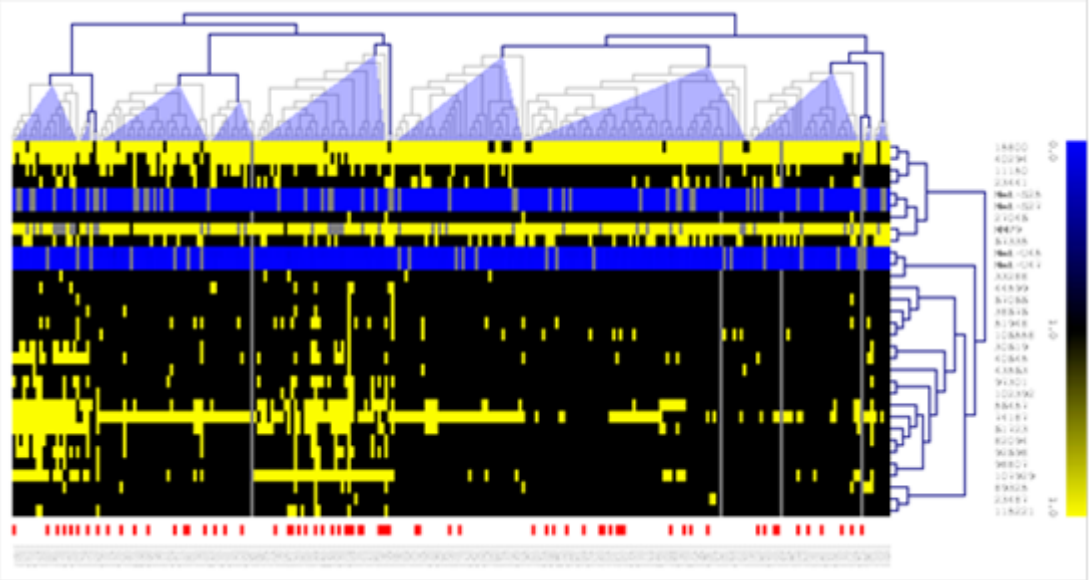

Figure 10. Clustering of Ingenious HyperCare patients based on expression levels of the five molecular markers.

A second important task related to WP7 was the analysis of the **health economic impact** of biomarker testing in the assessment of cardiovascular risk. This work has been led by the team at the University of Glasgow.

The analysis was based on the Scottish CVD Policy model to estimate the discounted health and cost outcomes associated with different methods of prioritisation. This is a decision-analytic model which predicts life expectancy, quality-adjusted life expectancy, and cost outcomes for individuals based on their ASSIGN risk factors. It currently exists as two extensive Microsoft Excel documents, one for males and one for females. The risk factors of a number of individuals from a large dataset were entered into the model and changes to health and cost outcomes were recorded. We have used the proteomic classifier HF1 developed in WP3 for modelling. HF1 has been shown to be a significant predictor of primary non-fatal CHD events, even when traditional risk factors are controlled for. Due to the novel nature of HF1 as a CVD risk factor, few large datasets exist which are able to estimate the hazard ratio associated with increases in HF1 score for non-fatal CHD events. It was necessary to obtain such a hazard ratio in order to estimate the effect of prioritisation based on HF1 score. Access was provided to the FLEMENGHO cohort for this purpose.

It was assumed that the cause-specific hazard for an individual with a mean HF1 score would be equal whether or not the covariate was not included in the model. This hazard would then increase or decrease in accordance with hazard ratios associated with HF1 obtained from the Gompertz regressions run on the FLEMENGHO dataset. In order to ensure the cause-specific hazard of an individual with mean HF1 score was comparable with and without the HF1 biomarker included in the hazard functions, the linear predictor's constant term β_0 (which independently affects underlying hazard) was calibrated. The updated Scottish CVD Policy Model was used to simulate the

effect that the giving of statins to different groups of people within this cohort would have on population life expectancy.

Implementing HF1 into the ASSGIGN score resulted in population-level outcomes associated with implementing biomarker testing and treating to the ASSIGNBIO score compared with using the traditional ASSIGN score. Sensitivity analysis suggested that an ASSIGNBIO score of 9.6% would lead to a similar number of individuals being prioritized for preventive therapy as an ASSIGN score of 10%. At a testing cost of £350, this policy would be dominated by current practice. It would incur additional costs while leading to a reduction in QALYs. If there were no testing costs, the ASSIGNBIO 10% strategy would save around £10,500,000. However, this would equate to a cost saving of around £3,500 per lost QALY, far below a reasonable cost-effectiveness threshold in any high-income country. However, an 81-94% reduction in costs (from £350 per patient) would result in the ASSIGNBIO testing strategy being cost-effective compared to current practice.

More detailed analyses have been performed throughout the course of the project. However, already the above data demonstrate that significant reductions in costs are required for widespread implementation of novel biomarkers to be economically sustainable. Such cost reductions have been seen for the majority of biomarkers in clinical use over a longer period of time but in the immediate term cost savings for health systems cannot be expected.

3.2.8 Work package 8: Management and coordination

WP8 provided the infrastructure for project management and coordination and was led by the team at **KITE Innovation Europe** together with the project coordinator.

Management activities focused on monitoring the project results and adherence to timelines and milestones. Timely submission of deliverable reports was another key task of the Work Package. Coordination benefited from close monitoring of open issues via regular meetings / teleconferences between the coordinator & project manager and the different project teams, organization of a Consortium teleconference and a Consortium meeting, monitoring of the financial and technical status of the project via internal updates, milestones and deliverables progress tracking and constant exploring of potentials for continuation of the project after its end via new collaborations within and beyond the Consortium.

3.2.9 Work package 9: Dissemination and exploitation planning

WP9 played a central role for the Consortium as EU-MASCARA was set up in response to an SME targeted call. Dissemination and exploitation, however, played a role for both the SME and the knowledge partners and more details will be provided in section 4 of this report. We will at this stage focus on key features related to WP9 which was led by the team at **KITE Innovation Europe**.

Key achievements in the area "dissemination"

1. Truly exceptional publication record: 174 publications at the time of writing this report (July 2016).
2. The EU-MASCARA Consortium organised a kick-off meeting in Glasgow followed by 4 annual Consortium meetings in Vienna, Leuven, Milan and Glasgow.
3. We had EU-MASCARA dedicated symposia at the European Meeting on Hypertension and Cardiovascular Protection on two occasions.
4. We published 4 E-zine editions i.e. our annual newsletter, on our EU-MASCARA website.
5. We presented EU-MASCARA to the UK industry sector at the Industry day of the University of Glasgow's College of Medical, Veterinary and Life Sciences.

Key achievements in the area "exploitation"

1. KITE distributed and evaluated an exploitation planning questionnaire at M36. Information collected provided a baseline to understand the exploitation potential for each SME in the remaining months of the project and in the post project situation.
2. Project management endeavoured to maintain the "voice of the SMEs" within the dialogue of a Consortium environment, whose centre of gravity firmly lies with the clinical research partners.
3. The EU-MASCARA Consortium kept monitoring progress with the SMEs to support the identification of exploitable opportunities.
4. KITE have been leading the process to monitor and capture the new scientific knowledge gained from the project. We have evaluated the publications made by the clinical research partners by Impact Factor and H values. We also sought to obtain a summary from those partners of how they intend to capitalise on the results from their most important publications.

3.3 Summary of the main S&T results/foregrounds and further analysis strategies

The EU-MASCARA project has evaluated a large number of biomarkers in a comprehensive range of cardiovascular conditions.

For this report we have selected a number of specific results that are related to specific biomarkers and specific disease areas in order to illustrate the extent of the work. Beyond the currently available data it should be noted that the Consortium will continue to collaborate beyond the funding period. Firm links between partners have been established and shared datasets, access to common resources and expertise and further joint grant applications will continue to feature in the future.

Data that have been generated as part of the project and will be further exploited beyond its lifetime include:

- Profiles of inflammatory markers in the FLEMENGHO cohort will be analysed cross-sectionally in relation to specific cardiovascular phenotypes and prospectively for changes in these phenotypes and development of cardiovascular events.
- Metabolomic profiles in women who had pre-eclampsia and are at increased cardiovascular risk will be explored. The data have been generated as part of the studies in the Generation Scotland – Scottish Family Health Study within EU-MASCARA.
- Longitudinal and integrative analysis of heart failure events in the Generation Scotland – Scottish Family Health Study within EU-MASCARA beyond the currently presented cross-sectional data.

4. Potential impact

The EU-MASCARA project aimed to validate biomarkers for assessment of cardiovascular risk for future use in clinical practice. The Consortium generated a large number of data that were disseminated through various channels during the course of the project and expected to produce further dissemination activities beyond the lifetime of EU-MASCARA. The potential impact of the project can be assessed by the exploitation strategies that have been developed by EU-MASCARA partners based on the project's results.

In this section we will first highlight the dissemination activities and then provide examples of exploitation strategies that will clearly demonstrate the impact of the project for the citizens in Europe.

4.1 Dissemination of project results

4.1.1 Dissemination to the scientific community

A total of 174 publications derived from the project at the time of writing of this report (July 2016). Some of the publications were published in the highest ranking journals (based on the 2014 Journal Impact Factor) in the field of cardiovascular sciences:

- Journal of the American College of Cardiology - 16.50 IF / 3 publications
- European Heart Journal – 15.20 IF / 8 publications
- Circulation – 14.43 IF / 3 publications
- Circulation Research – 11.02 IF / 4 publications

The overall distribution of publications grouped by Impact Factor is shown in Figure 11.

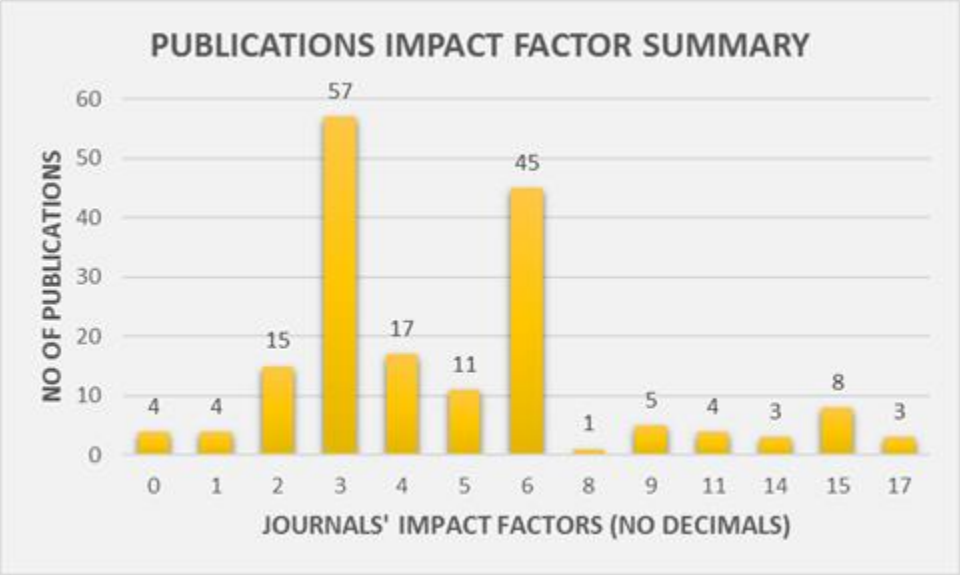

Figure 11. Impact Factors range in rounded up numbers and the number of publications in each range.

The Consortium produced a steady output throughout the duration of the project. This is in keeping with the call text that requested available samples, established techniques and biomarkers that are good candidates for further validation studies. The required infrastructure was therefore available right from the start of the EU-MASCARA project and output has been generated constantly over the years (Figure 14).

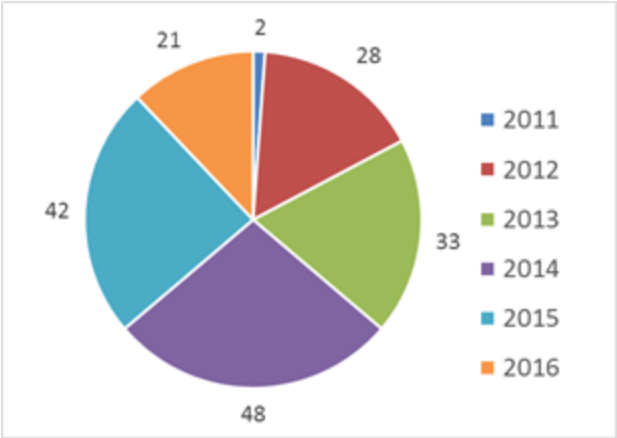

Figure 14. Number of published scientific papers that derive from EU-MACARA over the lifetime of the project (2011-2016).

In addition to published papers Consortium partners presented EU-MASCARA data with a total of 140 oral and 49 poster presentations at national and international conferences. These include some of the most renounced meetings in the CVD field such the congresses of European Society of Hypertension and European Society of Cardiology, the annual meetings of the International and the American Societies of Hypertension, the ERA-EDTA and the ESAO conferences, the Artery Research meetings, etc. It is important to say that EU-MASCARA are presenting their results in these events almost every year. In addition, they often present data collectively so the presence of the project as a whole is always strong.
From the 140 presentations the 100 derived from the partner's collaborative work and were presented as such. The same rate stands for posters (30 out of 49) and publications (120 out of 174).

Five scientific events have been organized by the partners in this period around subjects relevant to the project where EU-MASCARA featured prominently and collaboration between consortia was discussed.

There was also an EU-MASCARA thematic workshop "BIOMARKERS OF CARDIOVASCULAR RISK: THE EU-MASCARA PROJECT" organised during the 25th European Meeting on Hypertension and Cardiovascular Protection (2015, Milan, Italy). The workshop was chaired by Profs A.F. Dominiczak, C. Delles & A. Zanchetti. It involved 7 presentations by the EU-MASCARA partners and discussion sessions and received extremely positive feedback by the audience.

4.1.2 Dissemination to the industry

EU-MASCARA was an SME-targeted project with significant contributions by its SME partners. In addition to these existing links to industry the Consortium reached out to other businesses and consumers. Two prominent examples demonstrate these activities:

1. EU-MASCARA was presented (by both GLA and KITE) to the UK medical industry during the GLA College of Medical, Veterinary and Life Sciences Industry Day 2015 (September 2015).
2. MOS acknowledged the project contribution in a leaflet they produced at M53 for their KardiOM Test.

4.1.3 Dissemination to the general public

The wider public was kept updated on the project progress mainly via the EU-MASCARA website. The abstracts of all the project publications feature in the relevant section of the website, while in the news section, the partners' activities and the most promising project findings are presented in lay terms. Our website shows an average of 20 visitors per day having about 30 sessions of about 20 seconds each. This show a constant interest on our work and a well invested time in exploring the website content.

An article was published in Glasgow local popular press in the context of the World Hypertension Day and press releases were issued on occasions throughout the lifetime of the project. The final E-zine was disseminated via the EU-MASCARA website and the partners' network.

4.2 Exploitation and exploitation planning

EU-MASCARA has provided extensive opportunities for each of the SME partners to advance their business objectives; progress is reported below for each SME. Nevertheless, one of the outstanding major areas of common interest between the SME partners is the desirability of accessing validation cohorts to convert promising results from EU-MASCARA into commercially exploitable products and services. Although it has not been possible to make further suitable cohorts available within the timescale of the project, the roadmap for each SME to achieve this has been the subject of considerable intellectual capital. The challenge of accessing validation cohorts is not unique to EU-MASCARA.

4.2.1 SME partners

The following activities were undertaken with respect to SME exploitation:

1. Understanding of the Exploitation baseline
    i. Collection of information to provide a baseline from which to understand the exploitation potential for each SME in the remaining months of the project and in the post project situation
    ii. Review of competitive market information to assess the external influences that impinge upon the exploitation plans of each SME
2. Progress monitoring with the SMEs to update the baseline understanding and to continue to support the identification of emerging exploitable opportunities.
3. Maintaining the "voice the SMEs" at all opportunities within the context of the Consortium communication processes and meeting schedule.

Below the partners have summarised the impact of the project on exploitation strategies of SNME partners in the following sections.

Emergentec

The primary objective in EU-MASCARA has been to enhance the evidence base and credibility of their system biology platform to contribute to biomarker discovery. Emergentec have been focused on published data/data provided by EU-MASCARA partners to develop their proprietary methodology and on studies to identify drugs interfering with the processes identified as relevant in the pathophysiology of left ventricular hypertrophy. A final publication on the findings regarding molecular processes, biomarkers, and drugs interfering with these processes has been prepared. Following previous publications Emergentec are positive about the benefits of the EU-MASCARA project to the development of their research models and expect that the ongoing analysis will deliver commercial opportunities.

Emergentec's initial focus within the project was to identify molecules that are biomarkers of patients with CVD but the project has helped them establish that the best use of the Emergentec platform will be to target molecules that had a higher probability of identifying a patient's progression of CVD.

The project has given them an opportunity to gain valuable research based experience, lessons and insights which have added to their understanding of how to focus their platform and skills. It has also improved the functionality and validity of Emergentec's platforms and models. This in turn feeds into improvements in their commercial offerings and has altered their strategic focus. As a consequence of their involvement in EU-MASCARA their primary focus is now concentrated on assisting big biotech and pharma company research projects. Their work within EU-MASCARA has translated into improved marketing and an increase in the commercial opportunities for Emergentec.

ACS Biomarker BV

The main task of ACS Biomarker was to measure microRNAs in cardiovascular cohorts with follow-up long enough to draw conclusions about the predictive efficacy of the selected microRNAs. ACS Biomarker joined EU-MASCARA with background IP for the diagnosis (and potential prognosis) of heart failure. The goal of the project was to acquire definitive validation data of their miRNA biomarkers in heart failure cohorts. Whilst this proved to be an out of reach goal due the delay in access to suitable cohorts, the final period of the project involved the measurement of microRNAs in the Aldo-DHF cohort (section 3.2.2 of this report). Although this is a heart failure cohort, the commercial exploitation potential of the results is not guaranteed as the cohort exclusively consists of patients with diastolic heart failure. ACS Biomarker expect to use the results of this study to continue planning the eventual commercial exploitation whilst continuing to search for further opportunities and cohorts to secure definitive results.

mosaiques diagnostics

mosaiques diagnostics have gained specific exploitation opportunities from the EU-MASCARA project:

1. mosaiques diagnostics have developed a coronary artery disease predictive test from analysis of EU-MASCARA cohort samples which provide a 7-year prediction window (DiaPat® KardiOM Test).
2. mosaiques diagnostics feed data into their subsidiary DiaPat GmbH who are focused on commercialising mosaiques diagnostics' range of proteomic tests. DiaPat GmbH markets successfully new diagnostic tests in Europe for chronic renal diseases (including diabetic nephropathy), bladder cancer, prostate cancer, cholangiocarcinoma, coronary artery disease, heart failure, ureteropelvic junction obstruction in neonates and for early detection of graft-versus-host disease, based on the technology used in EU-MASCARA.
3. These products are currently commercially available in Germany for a cost of €430 per test. A more limited range of tests is available in the UK. MOS have continued to refine this assay throughout the course of the EU-MASCARA project.

Randox Testing Services

There have been delays in access to samples for biomarker measurements in the first part of the project but the final period of the project featured a significant utilisation of the sample test capability of Randox Testing Services. They have completed the analysis of the samples of the cohorts they received using a number of their commercially available multiplex biochip assays.

The volume of the data generated from these studies is considerable. It has not proved possible to complete its full analysis and consequently it has not been possible to develop a novel biochip before the end of the project. The full commercialisation of such a biochip will require a validation process using independent cohorts. However, Randox Testing Services remain committed to the goal of reaching a positive commercial outcome and will participate in further post project work that may bring this aspiration to fruition.

As a spin off Randox Testing Services' work within the EU-MASCARA project, predominantly due to the fact that cytokine assays were chosen, their work has generated a number of projects related to sample handling. The data on freeze-thaw cycles presented in this report (section 3.2.5) are one such outcome for Randox Testing Services where they now have a much more detailed understanding of fluctuations and variability of individual cytokines.

4.2.2 Clinical Research partners

Project management led by KITE Innovation Europe have been leading the process to monitor and capture the new scientific knowledge gained from EU-MASCARA. In this process KITE took two actions:

They evaluated the publications produced by EUMASCARA by Impact factor and H values. These data are presented in section 4.1.1 of this report. Examples of high ranking publication in the Journal of the American College of Cardiology (2015 Impact Factor 17.76) include:

- ***Circulating Biomarkers of Myocardial Fibrosis: The Need for a Reappraisal.***
  López B, González A, Ravassa S, Beaumont J, Moreno MU, San José G, Querejeta R, Díez J.
  J Am Coll Cardiol 2015;65:2449-56

- ***Myocardial collagen cross-linking is associated with heart failure hospitalization in patients with hypertensive heart failure.***
  López B, Ravassa S, González A, Zubillaga E, Bonavila C, Berges M, Echegaray K, Beaumont J, Moreno MU, San José G, Larman M, Querejeta R, Díez J.
  J Am Coll Cardiol 2016;67:251-60.

- ***Prognostic Relevance of Gene-Environment Interactions in Patients With Dilated Cardiomyopathy: Applying the MOGE(S) Classification.***
  Hazebroek M, Heymans S.
  J Am Coll Cardiol 2015;66:1313-23.

In the following paragraphs we will highlight a number of publications that derived from the project and indicate their impact and exploitation potential.

KITE have sought to obtain a summary from the clinical research partners of how they intend to capitalise on the results from their most important achievements either already published or under preparation.

UNIVERSITY OF GLASGOW

Differential expression of microRNA-206 and its target genes in preeclampsia.
Akehurst C, et al., J Hypertens 2015;33:2068-74.

This paper demonstrates a dysregulation of microRNAs in the development of pre-eclampsia. The target genes of miR-206 are critically involved in vasculogenesis and constitute biomarkers for other cardiovascular diseases. This paper adds another building brick to explaining why pre-eclampsia is considered a cardiovascular risk factor. EU-MASCARA gave the opportunity to study the long-term CV risk of women with pre-eclampsia and contribute to the awareness of pre-eclampsia as a gender-specific CV risk factor, a fact that is now also acknowledged for example by the American Heart Association.
Knowledge exploitation: EU-MASCARA has given an opportunity to establish the GLA team as a player in pre-eclampsia research. International collaborations (Prof. Markus Mohaupt, Berne; Dr Ralf Dechend, Berlin; Dr Louise Pilote, Montreal) have evolved from this.

Proteome-based systems biology analysis of the diabetic mouse aorta reveals major changes in fatty acid biosynthesis as potential hallmark in diabetes mellitus-associated vascular disease.
Husi H, et al., Circ Cardiovasc Genet 2014;7:161-70.

This paper introduced pathway analysis strategies that are key for integrative data analysis in WP7 and is collaborative work between GLA, EMG and MOS. Even if this is a study in a rodent model the principles of such analysis are similar to human data.
Knowledge exploitation: GLA has established integrative data analysis that has enabled them to secure further grant funding from the EU (e.g. sysVASC) and attract postgraduate students to Glasgow. The tools have been used to analyse the interplay between biomarkers in EU-MASCARA and other datasets.

Impaired renal function impacts negatively on vascular stiffness in patients with coronary artery disease.
Rossi SH, et al., BMC Nephrol 2013;14:173.

This paper was one of the first results from cross-sectional data analysis in EU-MASCARA. It showed that even in patients with very advanced vascular disease biomarkers can provide additional information about the exact levels of their vascular phenotypes.
Knowledge exploitation: GLA is now in a position to study links between biomarkers and specific vascular phenotypes and have applied this knowledge to conditions such as pre-eclampsia and diabetic kidney disease (in collaboration with the FP7 project "PRIORITY") to achieve a better understanding of the specific links between vascular disease and a range of pathophysiological principles.

FIMA

Biomarkers of cardiomyocyte injury and stress identify left atrial and left ventricular remodelling and dysfunction: A population-based study.
Ravassa S,et al., Int J Cardiol 2015;185:177-85.

The findings of this collaborative work between FIMA, K.U. Leuven and GLA reported in this paper expand the information on the potential diagnostic capacity of the combination of the serum biomarkers high sensitivity cardiac troponin T (hs-cTnT) and the amino-terminal pro-brain natriuretic peptide (NT-proBNP), not only for the detection of left ventricular structural and functional remodeling, but also identifying subclinical left atrial morphological abnormalities.
Knowedge exploitation: These results contribute to the search for pre-screening diagnostic tools in subjects with suspected subclinical left ventricular and left atrial alterations.

Association of cystatin C with heart failure with preserved ejection fraction in elderly hypertensive patients: potential role of altered collagen metabolism.
Huerta A, et al., J Hypertens 2016;34:130-8.

In patients with heart failure, cystatin C may be a biomarker associated with diastolic dysfunction and alterations in collagen metabolism, independently of renal function. The excess of cystatin C might contribute to diastolic dysfunction in heart failure patients by facilitating myocardial accumulation of pro-fibrotic factors.
Knowledge exploitation: The data draw further attention to the link between renal and cardiac diseases. Altered collagen metabolism is a feature that is also the basis for mosaiques diagnostics' urinary proteome-based biomarkers which have also been applied to both cardiac and renal disease. The results feed into future biomarker development in particular at mosaiques diagnostics.

Myocardial collagen cross-linking is associated with heart failure hospitalization in patients with hypertensive heart failure.
López B, et al., J Am Coll Cardiol 2016;67:251-60.

Excessive myocardial collagen cross-linking identifies patients with prevalent heart failure of hypertensive aetiology at risk of hospitalisation. In addition, our results indicate the potential usefulness of the serum C-terminal telopeptide of collagen type I (CITP):matrix metalloproteinase (MMP)-1 ratio to identify these patients.
Knowledge exploitation: FIMA is planning to further confirm the diagnostic and prognostic utility of these biomarkers alone, in combination, or implemented by other circulating molecules, mainly related with collagen metabolism, that may provide complementary pathophysiological information. In order to do so, a multidisciplinary team between FIMA and the Clinic University of Navarra has been established to carry out this task, contemplating the new recruitment of patients with or without symptoms of heart failure and including the development of follow-up protocols.

INCLIVA

Prognostic value of microalbuminuria during antihypertensive treatment in essential hypertension.
Pascual JM, et.al, J Hypertens 2014;64:1228-34.

This work provides support for the role of urinary albumin excretion as prognostic factor for CVD. A total of 2,835 hypertensive subjects were in the absence of previous CVD were followed up for a median of 4.7 years. Urinary albumin excretion was measured yearly during follow up. Events during follow up were correlated to urinary albumin excretion. Persistence or new development of microalbuminuria increase the risk of cardiovascular events independently of other cardiovascular risk factors.
Knowledge exploitation: INCLIVA plans to introduce the assessment of microalbuminuria in hypertensive patients under therapy as a proxy of the effect of anti-hypertensives beyond lowering blood pressure levels.

Genomic and metabolomic profile associated to microalbuminuria.
Marrachelli VG, et al., PLoS One 2014;9:e98227.

New statistical methodology for patient stratification in microalbuminuria was applied by combining metabolomics and genomics in 1,500 individuals of a general population study. Using this new approach, we identify two new genotypes with microalbuminuria resistance and predisposition. The

identification of these genotypes opens new potential ways for better understanding the disease and for early identification of individuals at risk.
Knowledge exploitation: INCLIVA plans to design new experimental research for better understanding of the potential role of these genes in the pathogenesis of microalbuminuria and hypertension. They will continue to explore the possibility of including these genes in the panel of polymorphisms regularly explored in cardiovascular risk clinical research studies.

The nutrigenetic influence of the interaction between dietary vitamin E and TXN and COMT gene polymorphisms on waist circumference: a case control study.
Mansego ML, et al., J Transl Med 2015;13:286.

High waist circumference is a cardiovascular risk factor. This study shows for the first time that genetic variation in oxidative stress related genes can modulate waist circumference in relation to vitamin E intake. The association between two genotypes and waist circumference in a Spanish population can be influenced by vitamin E intake.
Knowledge exploitation: In the future, we may request genotyping of these polymorphism in individuals with high waist circumference before suggesting vitamin E or antioxidants interventions.

UMA

Macrophage microRNA-155 promotes cardiac hypertrophy and failure.
Heymans S, et al., Circulation 2013;128:1420-32.

UMA's research in both a preclinical and clinical setting has led to novel insights. The mainly preclinical work in microRNA-155 has improved the insight in the pathophysiological mechanisms at play during either viral myocarditis or hypertension which are common diseases in humans. The above mentioned paper has generated data that supports the causative significance of inflammatory signalling in hypertrophic heart disease and demonstrate the feasibility of therapeutic microRNA targeting of inflammation in heart failure.

Matricellular proteins and matrix metalloproteinases mark the inflammatory and fibrotic response in human cardiac allograft rejection.
Vanhoutte D, et al., Eur Heart J 2013;34:1930-41.

We have demonstrated that inflammatory signalling not only occurs through cardiomyocytes or inflammatory cells, but is an interplay between different cell types including resident cells from the extracellular matrix. These novel insights have led to the translational paper mentioned above, indicating that certain matrix-related proteins (synd-1 and MMP-9) may act as a decision-making tool to discriminate rejecting from non-rejecting hearts.

Prognostic relevance of gene-environment interactions in patients with dilated cardiomyopathy: applying the MOGES classification.
Hazebroek MR, et.al, J Am Coll Cardiol 2015;66:1313-23.

Clinical research led by the University of Maastricht has evaluated gene-environment interactions in patients with dilated cardiomyopathy (DCM), a typical phenotype resulting from viral myocarditis or hypertension. These data indicated that although a genetic predisposition plays an important role, more is needed to develop DCM as not all patients with a genetic mutation will progress towards DCM. Interestingly, it appeared that the combination of triggers, i.e. a genetic mutation and a virus infection, was associated with a poor outcome. These results will lead to new insights regarding treatment and perhaps more importantly, prevention of DCM.

Knowledge exploitation (for all 3 papers above): Future strategies have already been initiated, focusing further on the translational aspect of the above mentioned results. An example is the use of patient-derived pluripotent stem cells, differentiated into beating cardiomyocytes and the mechanisms at play during viral infection.

UNIMIB

Variant on chromosome 9p is associated with left ventricular mass: results from two cohorts of essential hypertensive individuals
Cristina Mennia, et al., J Hypertens. 2012 Nov;30(11):2144-50 ,

Does the 9p region affect arterial stiffness? Results from a cohort of hypertensive individuals
Francesca Cesana et al., Blood Press. 2013 Oct;22(5):302-6

New knowledge for UNIMIB derived mainly from their research on arterial stiffness and arterial properties. They investigated three fields: structure, function and clinical implications of arterial properties. In particular, the structure was investigated thought biomarkers. They found interesting results with Annexin, cystatin C (175 consecutive hypertensive patients with fairly controlled BP values were compared to 175 healthy controls). The preliminary data showed an overexpression of Anx A5 in patients with arterial organ damage. A significate correlation with PWV (R=0,11; p= 0,04) was found while preliminary data showed that AnxA5 levels had a relationship with arterial stiffness in hypertensive and healthy subjects. They investigated also the role of Cystatin C in a population of 480 normotensive subjects free of kidney disease (KD) and cardiovascular disease (CVD). Cystatin C increase seems to well reflect arterial damage also in preclinical disease, it could be a good marker of initial atherosclerotic process.

Knowledge exploitation: Based on the above UNIMIB can speculate that Arterial Stiffness is associated with: Markers of activation and inflammation, Genetic factors, Markers of Hemostasis and others (like VEGF). Arterial Stiffness is involved in different clinical conditions: HT, diabetes and Metabolyc Syndrome, Psychology, Cancer and HIV and their treatments. All these conditions lead to high cardiovascular risk. The possibility to identify early biormarkers could help to better understand these pathologies and could help the choice of specific therapy and approach for each patient. UNIMIB plans to continue putting their effort in exploring the arterial system as a whole organ that shows specific biomarkers and multidisciplinary involvement.

RWTH

Identification of the Vasoconstriction-Inhibiting Factor (VIF), a Potent Endogenous Cofactor of Angiotensin II Acting on the Angiotensin II Type 2 Receptor.
S. Salem, et al., Circulation 2015;131:1426-34.

RWTH aimed to examine proteomic and metabolomic markers together with markers of cardiac remodelling to study their incremental diagnostic and predictive value for risk assessment of cardiovascular diseases in the general population and especially in patients with chronic kidney disease within the context of EU-MASCARA. The present paper describes a novel vasoactive factor that was discovered from proteomic work in EU-MASCARA.
Knowledge exploitation: The biomarkers and mediators identified by RWTH within EU-MASCARA Consortium provide the unique opportunity to predict the risk of development and/or progression of CVD as well as offers the highly relevant opportunity to develop new strategies and/or therapeutic

strategies and drugs for combating these conditions. Based on their results of EU-MASCARA, RWTH are developing a therapeutic drug for the treatment of hypertension and a therapeutic and/or preventive drug for vascular calcification in CVD patients.

KU LEUVEN

The most important achievements by KULEUVEN are listed below and the publications related to are annexed at the end of this summary:

1. In 2012, KUL in association with MOS did a case-control study and identified a specific signature of breakdown products of markers in urine indicative of decreased heart function.1. Subsequently, these markers were validated in the general population2 and shown to predict adverse health outcomes,3 including decline of renal function,4 over and beyond classical risk factors. The amino-acid building blocks of these urinary proteins were different in patients with decreased heart function compared with those predestined to develop renal dysfunction (submitted).

2. Matrix-Gla-Protein (MGP) is a protein activated by vitamin K, which is synthesised in the arterial wall of the kidney and heart. It is a strong inhibitor of arterial calcification. The level of inactive MGP in the circulating blood predicts all-cause mortality and cardiovascular complications, not including coronary heart disease.5 Moreover, KULEUVEN demonstrated in multi-ethnic population studies that renal function declines with higher levels of circulating inactive MGP,6 suggesting that activated MGP not only protects the arteries against calcification, but also helps in maintaining the filtration function of the kidney, which is a microvascular trait.

3. Collagen is a protein that provides scaffolding to many organs, including the heart and kidney. Worsening of the function of these organs is commonly associated with increased collagen deposition, a process known as fibroses. Within the framework of EU-MASCARA, KULEUVEN demonstrated that a decreased heart function is associated with circulating biomarkers indicative of cardiac fibrosis,7 injury of the heart muscle cells,7 and metabolic markers indicative of a less efficient utilisation of energy-providing substrates or less protection against oxidative stress and inflammation.8

4. Over the life cycle of EU-MASCARA, KUL in collaboration with other partners identified common genetic variants that in the general population increase the risk of hypertension,9 coronary heart disease10 or weakened pump performance of the heart.11 Mitochondria are organelles within living cells that are instrumental in generating the energy required for the normal function of organs. KUL observed that in the general population the heart function declines with a lower amount of mitochondrial genetic code in circulating white blood cells.11

Knowledge exploitation: KULEUVEN's work helped bringing to clinical practice some biomarkers, in particular the urinary proteomic profile, that hold great promise to make a more personalised approach to medicine possible.

CHARITE

New knowledge derived for CHARITE from their work on spironolactone in the Aldo-DHF cohort. Heart failure (HF) is a major health problem as patients with HF have a poor prognosis. More than 50 % of patients with the clinical symptoms of HF have a preserved ejection fraction (HFpEF). To date no treatment has been shown to reduce morbidity and mortality in those patients. The aim of the Aldo-DHF trial was to investigate the effects of aldosterone receptor blockade in stable HFpEF patients. A major component of the HFpEF pathophysiology is myocardial fibrosis, which is a result

of increased collagen synthesis. Higher levels of aldosterone are thought to be responsible for an increase in myocardial fibrosis due to an increase in collagen synthesis. CHARITE measured three biomarkers of collagen metabolism and demonstrated significant treatment-dependent changes in their occurrence over time. The findings allowed a better insight into the mode of action of spironolactone in HFpEF patients. Although the treatment with spironolactone did not alter the patients symptoms or quality of life it did have a significant influence on left ventricular geometry, diastolic function and neurohumoral activation. Additionally, microRNAs have been identified that can be used as potential new biomarkers for the early detection of HFpEF and as markers of disease progression. The collagen biomarkers possess prognostic value for the patients' risk prediction.

Knowledge exploitation: The above results are now in the process of getting published. In addition, it is planned to validate our findings in another HF cohort (DIAST-CHF), which includes patients with risk factors for diastolic HF or manifest chronic HF. This was in contrast a non-invasive study and most of the biomarkers are already analysed.

MHH

The Randomized Olmesartan and Diabetes Microalbuminuria Prevention (ROADMAP) Observational Follow-Up Study: Benefits of RAS Blockade With Olmesartan Treatment Are Sustained After Study Discontinuation
Jan Menne et al., J Am Heart Assoc. 2014; 3: e000810

MHH new knowledge derived mainly from their work on the ROADMAP cohort and in particular the investigation of clinical and cardiometabolic predictors for the development of microalbuminuria. In patients who developed microalbuminuria in the main study (duration of follow-up 3.2 years) significant alterations in several markers reflecting different stages in the inflammatory cascade of atheromatosis were identified. sFLT, TNFR-I, Angiopoietin II and ST2 were found to be significantly increased whereas TGFbeta-1, Osteopontin, Galectin-3, and C1qR1 were found to be significantly decreased in patients who developed albuminuria (no differences in 12 further markers tested were observed). Clinical and laboratory predictors for the development of albuminuria were identified, and the interaction between eGFR and albuminuria was delineated.

Knowledge exploitation: This work highlights the role of albuminuria as a cardiovascular risk factor by proving that its development is preceded by alterations in markers reflecting different stages in the inflammatory cascade of atheromatosis. This adds to the evidence regarding the significance of modifiable cardiometabolic risk factors for the development of albuminuria. This work will hopefully contribute to the timely recognition and treatment of risk factors associated with the development of albuminuria. The measurments of the markers in their OFU cohort has not competed yet. The results of the inflame study are planned to be published. One paper will deal with the decreases and one with the increases in the levels of the markers before the development of albuminuria.

AUX

Randomized controlled trials of blood pressure lowering in hypertension: a critical reappraisal.
Zanchetti A, et al., G. Circ Res. 2015 Mar 13;116(6):1058-73.

Sixty-eight blood pressure (BP)–lowering randomized controlled trials (defined as randomized controlled trials comparing active treatment with placebo, or less active treatment, achieving a BP difference, performed between 1966 and end 2013 in cohorts with ≥40% hypertensive patients, and

exclusive of trials in acute myocardial infarction, heart failure, acute stroke, and dialysis) were identified and meta-analyzed grouping the randomized controlled trials on the basis of clinically relevant questions: (1) does BP lowering reduce all types of cardiovascular outcome? (2) Is prevention of all outcomes proportional to the extent of systolic, diastolic, and pulse BP? (3) Have all classes of BP-lowering drugs been shown capable of reducing all types of cardiovascular outcome? (4) Is BP lowering beneficial when intervention is initiated at any grade (or stage) of hypertension? (5) Do BP-lowering randomized controlled trials provide evidence about systolic BP and diastolic BP targets of treatment? (6) Should BP-lowering treatment be preferentially addressed to patients in higher risk categories promising larger absolute treatment benefits? The results of these meta-analyses provide further support to current hypertension treatment guidelines by showing that BP lowering can significantly reduce major cardiovascular outcomes largely independent of the agents used, significant risk reduction is found at all hypertension grades (stages), and when systolic BP is lowered below a cut off of 140 mm Hg with some further reduction limited to stroke at systolic BP values just <130 mm Hg. Absolute risk reduction progressively increases higher is total cardiovascular risk, but this greater benefit is associated with a progressively higher residual risk, ie, higher treatment failures.

4.3 Assessment of achieved against anticipated impact

In the original proposal the EU-MASCARA Consortium outlined the potential impact of the work programme in four distinct areas. We will briefly summarise the achieved impact against these goals.

Complementarity to public health activities that aim to reduce the overall population risk of cardiovascular disease
EU-MASCARA has worked in concert with strategies to reduce cardiovascular risk. The project had a focus on studies in general population cohorts in order to translate results to the largest possible communities across Europe. Work within the project has addressed factors beyond molecular mechanisms of disease and studied for example the association between lifestyle factors and body weight, cardiovascular risk factors and biomarkers of CVD.

Improved cardiovascular risk prediction
A number of biomarkers that were validated and further developed within EU-MASCARA have been shown to improve cardiovascular risk prediction beyond traditional risk factors. In particular, the urinary proteomics derived biomarkers have fulfilled this goal and have already been commercialised.

Contribute to the development of personalised and predictive medicine
"Personalised medicine" or "precision medicine" feature strongly in malignant diseases and inflammatory diseases but less so in CVD. The complexity and variety of CVD with their multifactorial origins and the relatively long duration of development of CVD are reasons for the slow uptake of precision medicine in CVD. The EU-MASCARA Consortium has therefore systematically evaluated a large number of biomarkers that can contribute to better risk stratification and inform treatment decisions. The health economic analyses that were performed as part of the project provide further guidance in this respect.

Encourage SME efforts towards research and innovation with priority given to proposals demonstrating that research-intensive SMEs play a leading role.
It is evident from this report that without the expertise of SME partners the EU-MASCARA project would not have been able to deliver its results. SMEs involved in this project continue to collaborate with knowledge partners beyond the lifetime of the project and within other international consortia.

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