PROJECT FINAL REPORT

Grant Agreement number: 305739 Project acronym: RiskyCAD

Project title: Personalized diagnostics and treatment for high risk coronary artery disease

Funding Scheme: Small or medium-scale focused research project

Period covered: From 01/11/12 to 31/10/2017

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4.1 Final publishable summary report

1. Executive Summary

RiskyCAD aimed at (i) identifying lipidomic and miRNA biomarkers for high risk coronary atherosclerosis and use them alone or in combination with standard risk models to improve the identification of patients in high risk for myocardial infarction or death, (ii) unwinding mechanistic links between disease risk and biomarkers, and investigating rational therapeutic approaches, (iii) developing novel diagnostic and therapeutic strategies based on patient stratification and 'drug repositioning'.

The project has achieved its objectives. New lipidomic biomarkers were identified as independent predictors of cardiovascular disease death and reproduced in independent cohorts. Among them, four ceramides, ceramide (d18:1/16:0), Ceramide (d18:1/18:0), Ceramide (d18:1/24:0) and Ceramide (d18:1/24:1) were selected and used to develop a new clinical test for predicting cardiovascular disease risk. The test has now been advanced to clinical use both in the USA and Finland. New circulating fatty acid and miRNA biomarkers were characterized in plasma samples from the LURIC cohort that can form the basis for further studies towards that direction. A new risk stratification algorithm for stable coronary artery disease, Coropredict®, based on readily available laboratory parameters has been developed and validated in external cohorts. A computer program incorporating the scientific results and the interpretation of measurements of Coropredict® has been completed and is ready for further exploitation. This program can operate in the stand-alone modus, can be linked to information systems of laboratory service providers, and can be used as a conjunct to point of care devices in practices and pharmacies.

New human and mouse models of coronary atheroscleroris have also been developed. Induced pluripotent stem cells (iPS cells) from skin fibroblasts of high and low risk CAD patients were generated and differentiated into hepatocytes, endothelial cells and monocytes/macrophages. Lipidomics, transcriptomics and miRNA analyses and functional studies for selected iPSC lines have been performed. Mouse models of coronary atherosclerosis and plaque vulnerability based on perturbed shear stress, conditional deletion of scavenger receptor BI (SR-BI) or activation of the ER stress response have also been established, new disease mechanisms characterized and new disease targets identified.

Finally, through the use of the bioinformatics-based predictive analytics platform (COSS), scanning over 15,000 drugs (small molecules, biologics with pharmacological action) and 150,000 compounds,

drugs with repositioning potential for the treatment of high-risk CAD have been identified. Among them, Tranilast [N-(3,4-dimethoxycinnamoyl) anthranilic acid], an anti-allergic drug approved for use for asthma treatment in Japan, proved to be particularly effective for the treatment of atherosclerosis in experimental animal models and human cell culture systems.

In summary, RISKYCAD has led to the development of novel biomarkers and tools for the assessment of CAD risk, some of which were commercialized during the project, and the identification of novel targets and drugs with repositioning potential for CAD treatment.

2. Summary description of project context and objectives

The general aims of RiskyCAD were 1) to identify lipidomic and miRNA biomarkers for high risk coronary atherosclerosis and use them alone or in combination with standard risk models to improve the identification of patients in high risk for myocardial infarction or death, 2) to unwind mechanistic links between disease risk and biomarkers, and investigate rational therapeutic approaches, 3) to develop novel diagnostic and therapeutic strategies based on patient stratification and 'drug repositioning'. The programme has achieved all its objectives.

The genotyping of the LURIC cohort (based on the Illumina exome array) has been completed, yielding data on rarer variants for 2016 samples. Analyses have been performed and data have also been submitted to international meta-analysis consortia. The processing of the LURIC samples for circulating miRNAs in plasma has also been done, analyses performed and new miRNA biomarkers identified. A new risk stratification algorithm for stable coronary artery disease based on readily available laboratory parameters has been developed and validated in external cohorts. The analysis of the LURIC cohort also revealed an association of specific fatty acid subspecies with the prognosis of patients with and without coronary artery disease being further explored. An update on the LURIC cohort information has been completed. Data from the last follow-up questionnaire as well as data from individual medical records have been entered into the LURIC database.

The generation of induced pluripotent stem cells (iPS cells) from skin fibroblasts of high and low risk CAD patients has been completed. From the 18 individuals skin biopsies collected, a total of 28 acute, 53 stable, and 20 control iPSC lines were generated. Methodologies for their differentiation into hepatocytes, endothelial cells and monocytes/macrophages have been developed and optimized. iPSC-derived cell lines have been characterized in detail. Lipidomics, transcriptomics and miRNA

analyses for some of these differentiated iPSC lines have been performed. Functional studies were done using iPSCs differentiated into endothelial cells or monocytes/macrophages on the integrity of the endothelial layer or its phenotype.

New mouse models of coronary atherosclerosis and plaque vulnerability have been developed based on perturbed shear stress and conditional regulation of SR-BI or the ER stress response. Novel targets of coronary atherosclerosis and plaque vulnerability have been identified. The transcription factor IRF5 proved to be particularly interesting as it was found to promote necrotic core formation and larger lesion size in *Apoe*$^{-/-}$ mice.

Ceramide biomarker candidates identified in the Corogene study, reconfirming the LURIC study's data were validated in two additional cohorts, BECAC and SPUM-ACS and results published. Four ceramides, Ceramide (d18:1/16:0), Ceramide (d18:1/18:0), Ceramide (d18:1/24:0) and Ceramide (d18:1/24:1) were selected and used to develop a new clinical test for predicting cardiovascular disease risk. A validated analytical method ready for clinical application (in the setting of clinical laboratories) has been reported. The test has now been advanced to clinical use both in the USA and Finland. Mayo Medical Laboratories (MML, Rochester, Minnesota) licensed the ceramide lipid based test from Zora Biosciences Oy in Q2-2016 and started to commercially offer that test in the USA marker end of July 2016. In Finland, Zora Bioscience Oy licensed the ceramide based test to United Medix Laboratories (UML). UML is the largest private medical laboratory in the country and it is offering laboratory services to the majority of private and public clinics in Finland.

Finally, the COSS predictive platform was used to identify drugs of repositioning potential for the treatment of high-risk CAD patients. Among them, tranilast, an anti-allergic drug, has been very effective in treating experimental atherosclerosis in mice.

The scientific and administrative management of the project has been successfully implemented. Extensive dissemination activities have been performed (brochure, project folders, functional website, publications, lectures etc). Apart from the dissemination of the project results through a large number of scientific publications, the communication tools of the project have been presented in all possible occasions mostly in meetings with external audiences. Six project meetings took place within the duration of the project.

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

Discovery of lipidomic biomarkers and development of a new diagnostic test

During the project, new lipid biomarkers were identified. Among them, Ceramide (d18:1/16:0), Ceramide (d18:1/18:0), Ceramide (d18:1/24:0) and Ceramide (d18:1/24:1) were used for the development of a clinical diagnostic test based on a validated analytical method applicable to clinical laboratories. This has been published (Kauhanen D, Sysi-Aho M, Koistinen KM, Laaksonen R, Sinisalo J, Ekroos K. Development and validation of a high-throughput LC-MS/MS assay for routine measurement of molecular ceramides. **Anal Bioanal Chem**. 2016 ; May;408(13):3475-83). The test provides information on both plasma concentrations and ratios of these distinct ceramide species and its results are reported as a Ceramide Score having values from 0 to 12.

This new diagnostic test has now advanced to clinical use both in the USA and Finland. Mayo Medical Laboratories (MML, Rochester, Minnesota) licensed the ceramide lipid based test from Zora Biosciences Oy in Q2-2016 and started its commercialization in the USA market at the end of July 2016 (Figure 1). MLL covers thousands of hospital and doctor's offices across USA and is therefore able to offer this new cardiovascular outcome test very broadly.

The diagnostic test has entered the European market in October 2016 in Finland. Zora Bioscience Oy licensed the ceramide based test in Finland to United Medix Laboratories (UML). UML is the largest private medical laboratory in the country and it is offering laboratory services to the majority of private and public clinics in Finland. Further license agreements both in Europe and USA are under negotiation.

The USA the test has branded as the CERAM test: http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/65054

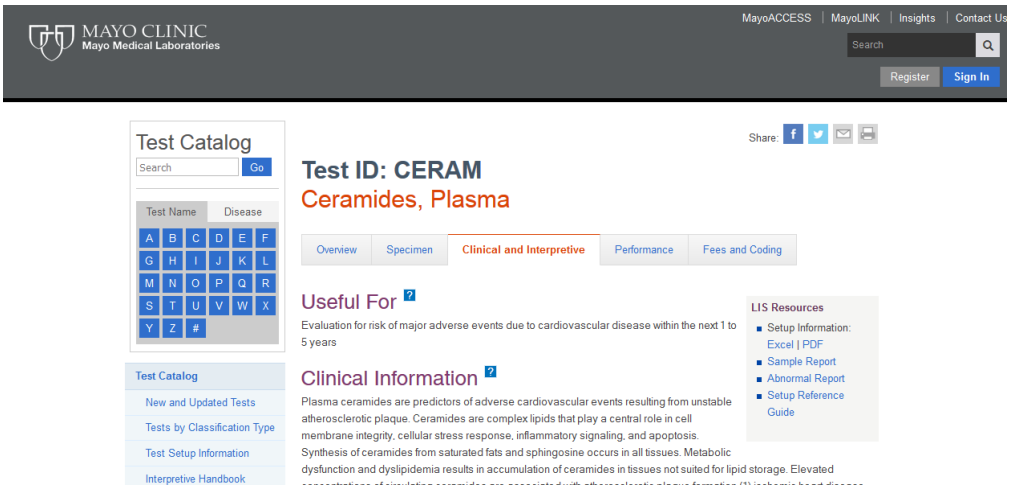

Figure 1. Commercialisation of new cardiovascular disease risk test with the name CERAM in the US (Mayo Medical Laboratories).

In Finland Zora Biosciences Oy has branded the test as the HERTTA-test (Figure 2 below; www.hertta.fi).

Figure 2. Commercialisation of the new cardiovascular disease risk test with the name Hertta in Finland.

HERTTA has been actively marketed both to public and medical doctors. Medical doctors have been approached directly in their offices and via advertisements in professional journals and webpages. Large public has been informed about the new test by media campaigns covering newspapers and social media including Facebook and twitter. Moreover, a major news channel picked and presented the test in their prime time program leading to very broad public exposure in Finland. Figure 3 below shows the dramatic effect of marketing and dissemination efforts that took place 04/2017 on commercial success and projected sales of the test.

Figure 3. Sales of the new cardiovascular disease risk test with the name Hertta in Finland.

Development of CAD risk prediction algorithms and tools for patient stratification

During RiskyCAD, we developed a novel proprietary multimarker algorithm for predicting cardiovascular risk in asymptomatic persons and in persons with pre-existing cardiovascular disease (CAD) named Coropredict®. To facilitate exploitation and acceptance of the CoroPredict risk engine in daily practice, we selected biomarkers already known to physicians, which are actionable by established medicines are automated and cost-effectively measured with a standardized laboratory system or with a commercially available point of care platform. Our first product, CoroPredict 1.0, thus consists of eight biomarkers that can be automated and cost-effectively measured with a standardized laboratory system or with a commercially available point of care platform (Table 1).

Table 1. Variables included in the CoroPredict algorithm

Variable	Remark
Age	
Gender	
LDL-C	No constituent of risk algorithm
HDL-C	No constituent of risk algorithm
HbA1c	Diabetes mellitus
Cotinine	Smoking
Cystatin C	Kidney function
Vitamin D	Lifestyle
NT-pro-BNP	Cardiac function
Troponin T	Cardiac function

Galektin-3	Fibrosis
ST2 / CRP / Fibrinogen	Chronic inflammation

The proprietary analysis algorithm Coropredict® integrates the results and reports an individual´s ten years risk of a fatal heart attack in the form of an aggregated "risk score" in a completely non-invasive fashion. Reporting and visualization of the laboratory results is patient-friendly and provides ‘competing’ risk scores for healthy people from the Framingham, PROCAM, or ESCHeartScore scores which are mostly based on clinical information and lack meaningful laboratory markers. A computer program incorporating the scientific results and the interpretation of measurements has been developed and is now available (Figure 4).

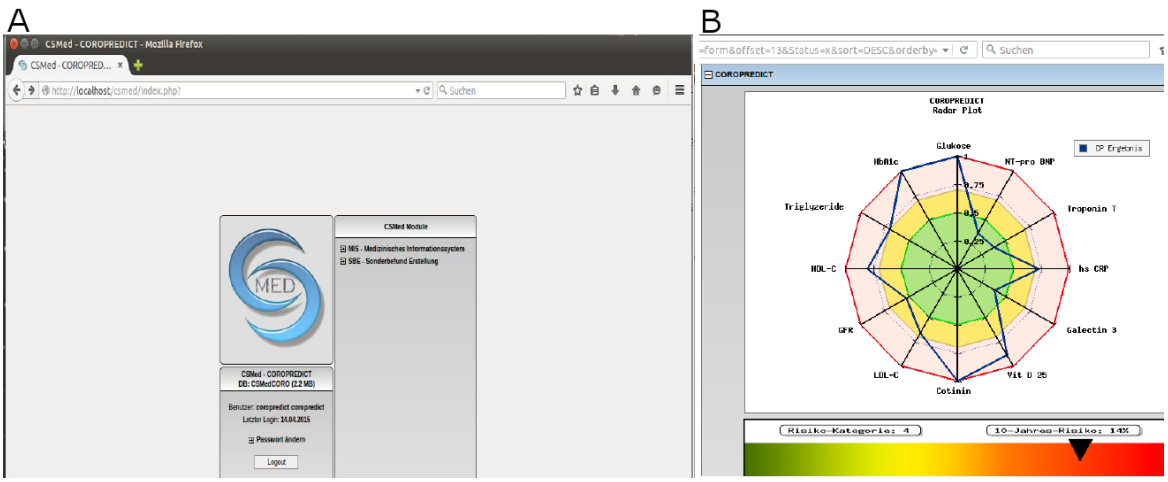

Figure 4. CoroPredict software module implemented in CSMED. Start screen (A) and example of radar chart showing individual parameters as well as overall risk score (B).

A comparative analysis of over 3000 patients from the LURIC study showed that CoroPredict 1.0 can identify patients at risk significantly better than individual biomarkers or the traditional risk scores by combining multiple biomarkers (Figure 5). The proprietary algorithm is a result of two decades of research and is based on an actual follow-up period of more than 10 years for clinical endpoints. The CoroPredict risk prediction score for patients with stable coronary artery disease has been replicated in another external cohort, the VIVIT cohort, that comprises 1001 consecutive Caucasian patients who were referred to elective coronary angiography for the evaluation of established or suspected stable CAD in Austria.

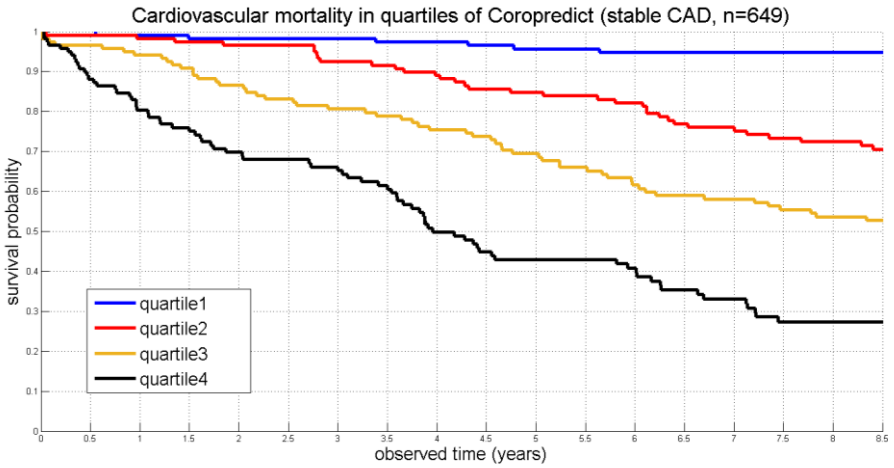

Figure 5. Association of CoroPredict score quartiles with cardiovascular mortality in patients with stable CAD.

A CoroPredict laboratory investigation is typically initiated by doctors interested in prevention (general practitioner or internist). The doctor performs the sample collection, sends the sample to the laboratory and discusses the diagnosis with the patient. His benefit is that he will be able to bind economically attractive privately insured and self-paying patients through cutting-edge medicine.
The benefit to the patient is that he can take appropriate measures (lowering cholesterol, healthier lifestyle, etc.) to reduce his risk of heart attack. By repeating CoroPredict measurements, the success of these measures can be quantified and the patient can be motivated to follow the treatment recommendations or proposals for changes in lifestyle permanently.

Discovery of microRNA biomarkers and biomarker signatures for high risk CAD

More than 2000 individual miRNAs are now known in humans and some have been associated with cardiovascular disease. Yet, solid evidence from large cohorts of patients is still lacking. By analysing a total of 999 citrate plasma samples and 751 EDTA plasma samples from patients of the LURIC study, we identified miRNAs that are linked to various phenotypes including all-cause mortality. A number of miRNAs identified were nominally with concordant directions of effect. By using the top three miRNAs, we constructed a miRNA score. Study participants with a higher miRNA score showed an increased mortality risk in Cox regression analyses adjusted for age, gender, LDL-C, HDL-C, TG, diabetes mellitus, hypertension, smoking, BMI, NT-proBNP, hsCRP, eGFR, ACE inhibitors, statins, coumarins, aspirin. The same was true when the analyses were restricted to only those study participants with stable CAD at baseline demonstrating the potential suitability of the miRNA

signature for risk classification of CAD patients.

Although these findings are relatively immature, they still constitute a major step forward towards the use of miRNAs as biomarkers for CAD. As next steps for their eventual exploitation, these new biomarkers will need replication in other unrelated cohorts and different trial designs, and development of assays for their measurement in routine clinical settings.

Development of new experimental (human and mouse) models of CAD

Innovative human model systems based on reprogrammed iPS cells have been developed. iPSCs generated from skin biopsies of high and low risk CAD patients were differentiated into hepatocytes, endothelial cells and monocytes, providing unique cell systems to study cAD. These carry the generic background and biological fingerprint of CAD patients and can thus be used for the unwinding of disease mechanists and the evaluation of new drugs. Reprogrammed iPSCs constitute a valuable tool to predict existing drug efficacy and toxicity in individuals, and direct treatment to individual patients in a truly 'personalized' manner.

Innovative animal models of atherosclerosis and CAD have also been developed. These address limitations of current animal models in terms of plaque vulnerability and time of development of atherosclerotic lesions. Mouse models of atherosclerosis based on perturbed shear stress provide an excellent system for studying the effects of low and oscillatory shear stress in plaque development and vulnerability. Mouse models enabling the conditionally regulated deletion of the SR-BI gene or regulation of the ER stress response provide further systems for investigating atherosclerosis and plaque instability. Both the human and mouse models thus provide new unique models for dissecting disease mechanisms and evaluating experimental therapeutics, offering opportunities for commercial exploitation.

New targets and drugs with repositioning potential for the treatment of CAD

Through the use of the bioinformatics-based predictive analytics platform (COSS), scanning over 15,000 drugs (small molecules, biologics with pharmacological action) and 150,000 compounds for their potential effectiveness in the treatment of coronary artery disease (CAD), seven drugs with repositioning potential for the treatment of high-risk CAD have been identified. Among them, Tranilast [N-(3,4-dimethoxycinnamoyl) anthranilic acid], an anti-allergic drug approved for use for

asthma treatment in Japan, was particularly effective in experimental animal models.
Therefore, the repositioning recommendations constitute promising drug candidates for the treatment of CAD with significant exploitation potential.

Promising new targets against which compounds can be developed have also been identified such as IRF5. The transcription factor interferon regulatory factor (IRF)-5 is a master regulator of inflammatory macrophage programming, controlling pro-inflammatory gene expression, efferocytosis and necrotic core formation during atherogenesis.

4. Description of potential impact, dissemination activities and exploitation of the results

4.1. Potential impact

The project has major implications for researchers in the field, the wider scientific community, healthcare professionals and the society in general. By discovering novel biomarkers and developing new diagnostic tests and algorithms for assessing CAD risk and identifying patients in need of treatment, RISKYCAD is likely to affect the way CAD patients are managed, reducing unnecessary treatment or implementing necessary treatment to the individuals most in need. By unwinding novel disease mechanisms, defining new targets and identifying drugs with repositioning potential for CAD, the project is likely to strengthen translational research activity in the field with significant impact to the pharmaceutical or biotechnology industry, and significant eventual benefit for the patient. Finally, by generating new human models of CAD based on iPSCs and new mouse models of CAD and plaque vulnerability, the project will support basic and clinical research in the broader area of cardiovascular diseases and plaque vulnerability, enabling new target identification and evaluation as well as drug screening. Overall, RISKYCAD has the potential to significantly impact patient care, healthcare professionals' mode of operation, academic and pharmaceutical research in the field, and policymakers, contributing to personalized medicine solutions and improving disease management, eventually benefiting the patient. The longterm effects will be better health with reduced morbidity, mortality and healthcare costs.

4.2. Dissemination activities

Widespread dissemination and communication of RiskyCAD's results has been a central target of the project (WP8). All partners have played an important role in the dissemination and communication activities. The **Dissemination** measures ensured that RiskyCAD's results would reach potential users and stakeholders, whereas the **Communication** activities aimed at reaching the general public.

A broad range of dissemination channels were developed and performed in order to achieve the highest level of dissemination of the project results within and outside the RiskyCAD consortium, during the lifecycle and after the end of the project (Table 2). The dissemination activities ensured that the project's results reach their potential users, increase the visibility of the project, facilitate a successful integration with stakeholders, allow networking and marketing the consortium and bridge the gap between researchers and the public. Dissemination activities started with the development of a common public visual identity for the project including an official logo, information brochure and project templates. The dissemination strategy also included scientific publications in high impact peer-reviewed scientific journals, regular presentations at scientific meetings and conferences, presentations, as well as internet based knowledge transfer to the scientific community and to the public.

- **Dissemination within the RiskyCAD consortium**

Regular consortium meetings are necessary for the prompt dissemination of results among the project partners. In RiskyCAD, scheduled physical meetings took place annually and a number of other web or physical meetings were organized at a consortium level and at a work package team level, as needed.

Effective internal communication was also ensured through the project's website (private area). A secure intranet system restricted to RiskyCAD members was developed. This functional site acted as a portal for all participants and as a repository for all project official and working documents and for other ethical, regulatory and gender policy documents.

The Management Team continuously updated information during the project duration, to ensure that the project run smoothly and that there was no information missing which could have an impact on the project. The website also provided information on major scientific advances and on scientific articles published by the RiskyCAD consortium.

- **Dissemination beyond the RiskyCAD consortium**

The main aim of the scheduled dissemination activities was to spread knowledge beyond the consortium and to promote the project by providing appropriate information to all targeted audiences. The European Commission's FP7 funding programme has been duly acknowledged in all dissemination activities and publications of RiskyCAD.

The target groups that have been identified were:

- **The academic community:** The exchange of knowledge with other research groups that are engaged in related research fields will be pursued. This was achieved through joint scientific publications, participation in conferences, invited talks and seminars and the RiskyCAD website public area.

- **Stakeholders:** This group consisted of the pharmaceutical industry, clinicians, as well as EU and national regulatory bodies. Awareness was raised on project results by networking with stakeholders, and providing relevant information for further applications. This was accomplished through the RiskyCAD website, talks, brochure, conferences and targeted direct contacts.

- **The public**: Public visibility and support was sought by raising awareness on EU investments in health research using RiskyCAD as a living example. The RiskyCAD website was used for this purpose.

An indicative list of the main dissemination activities undertaken by the consortium follows:

1. An internet website (http://www.riskycad.eu) has been created. The site contains in its public area basic information about the project, the consortium members, the scientific publications that have arisen from the project, the contact details of the coordinator etc. Project-related publications have been uploaded to the public area of the website. The RiskyCAD website will be active for five years following the end of the project. The coordinator has secured funding from other sources for the web hosting and domain purchase fees. A downloadable project's brochure (electronic version) has a prominent position at the front page of the website
2. RiskyCAD's logo was also made available to the partners for use in dissemination material. The logo was and is being used as the spearhead of the dissemination policy of RiskyCAD being the central element of the visual identity of the project. The logo appears in all supporting second line dissemination tools such as presentation templates.
3. Scientific Publications. A large number of scientific publications has arisen from RiskyCAD.

4. Brochure in hard copy and electronic format (Figure 6). A project's brochure was produced and a large number of brochures were distributed to the partners to be used as dissemination material in international conferences, within their scientific networks and other fora. An electronic version of the brochure has been uploaded to the website.
5. Project folders. Project folders with logo and contact details were also produced and distributed to enhance the visual identity of the project. The partners have been very keen on using the dissemination tools.

Table 2. Overview of RiskyCAD's main dissemination activities

Type of dissemination	Audience
RiskyCAD Web site : public and intranet	General Public and consortium members
RiskyCAD Logo, Brochure	General Public and stakeholders
Press releases	General Public, patient groups and stakeholders
Oral or poster presentation at international conferences	Medical and Scientific Community
Publications in high impact peer-reviewed journals	Scientists and Clinicians

Figure 6. The brochure of the RiskyCAD project that was produced and distributed around

4.3. Exploitation of the results

Since its preparation, RISKYCAD has had a strong translational focus aiming primarily at developing novel biomarkers and diagnostic tests for assessing CAD risk and guiding therapy. It has therefore integrated cross-sectoral academic expertise with four active SMEs and one industrial Partner in order to ensure efficient exploitation of the generated results. The SMEs involved are highly specialized in several key areas, ZORA on lipid biomarker discovery, CBC on miRNA biomarker discovery, BIOVISTA on 'drug repositioning', adverse effect profiling and cohort analyses, and genOway on disease animal models. The industrial Partner SYNLAB, being the largest diagnostic services provider in Europe, is a major potential end user of the project's foreground. The academic Partners, in turn, have previous experience in research result exploitation through collaborations or spin-off companies. Awareness on IP issues within the consortium has been raised while there have been frequent discussions about the foreground in RiskyCAD's regular meetings. All measures thus to identify, protect, manage and exploit foreground have been undertaken.

The most important exploitable foreground and the current status of its commercialization includes:

1. New lipidomic biomarkers and lipid-based diagnostic tests
2. New CAD risk prediction algorithms and tools for patient stratification
3. MicroRNA biomarkers and biomarker signatures for high risk CAD
4. New experimental (human and mouse) models of CAD
5. New targets and drugs for the treatment of CAD