



Project Final Report

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Name, title and organisation of the scientific representative of the project's coordinator:

Prof. Stefan Anker - Charité – Universitätsmedizin Berlin

Tel: +49 30 450 553 151

Fax: +49 30 450 525 951

E-mail: s.anker@charite.de

Project website address: www.sica-hf.com

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Section 1 – Final publishable summary report



Project title: Studies Investigating Co-morbidities Aggravating Heart Failure

Website: www.sica-hf.com

Contractors involved (SICA-HF consortium):

The project is coordinated by Prof. Stefan Anker (Partner 01) Charité - Universitaetsmedizin Berlin, Germany, Address of the Coordinator: Charitéplatz 1, 10117 Berlin, Germany.

Other partners and team leaders:

Partner 02: HULL, University of Hull, United Kingdom

Partner 03: WROC, Uniwersytet Medyczny Im Piastow Slaskich We Wroclawiu, Polen

Partner 04: IRCCS, San Raffaele S.p.A., Italy

Partner 05: MHH, Medizinische Hochschule Hannover, Germany

Partner 06: GABO:mi, GABO:mi Gesellschaft für Ablauforganisation :milliarium mbH & Co. KG, Germany

Partner 08: V.A. Almazov Federal Centre for Heart, Blood & Endocrinology, Russia

Partner 09: MSU, M V Lomonosov Moscow State University, Russia

Partner 10: CT, Inst. of Russ. Acad. of Med. Sci. Scientific Researchinst. of Card. of Siberian Dept. of Rams, Russia

Partner 11: CRC, Russian Cardiological Research and Production Complex, Russia

Partner 12: Golnik, Bolnisnica Golnik Klinicni oddelek za pljucne bolezni in alergijo, Slovenia

Partner 13: SCCS, Silesian Centre for Heart Diseases, Poland

1.1 Executive summary

SICA-HF, consisting of outstanding European heart failure clinicians and basic researchers, has accomplished to provide a common platform for the two different research teams. All partners of SICA-HF recruited more than 1462 patients with chronic heart failure, 199 patients with type 2 diabetes without heart failure, and 173 healthy control subjects. Thus, during the last year, a cumulative 91.4% of the target value was achieved for patients with chronic heart failure, 66.3% of the target value for diabetic controls, and 115.3% and thus over-achieving for healthy subjects. The data of all participating centers were collected and entered into the central database. As of this writing, 1469 of all chronic heart failure patients' visits, 255 of diabetic controls' visits, and 167 healthy controls' visits have been entered into the online database. In total, 171 patients have been reported to be deceased; the total number of hospitalizations is 2232. In addition, a total of 127 skeletal muscle biopsies and 92 fat tissue biopsies have been collected. Basic researchers were given the opportunity to share their views with clinicians to develop clinically relevant basic research questions. The pathophysiology in chronic heart failure as well as cardiac reorganisation and repair are highly complex. Developing successful strategies for cardiac tissue protection and repair therefore require a joint effort of experts on basic science on a vascular, molecular, and cellular, but also on clinical level. In addition, SICA-HF focussed on skeletal muscle function and wasting as well as on adipose tissue signalling. SICA-HF identified skeletal muscle wasting as a novel and frequent co-morbidity among patients with chronic heart failure. We found that patients with muscle wasting presented with reduced exercise capacity, decreased muscle strength, and advanced disease. A total of 19.5% of patients in this cohort with clinically relevant HF presented with clinical features diagnostic of muscle wasting, defined using the criteria suggested to diagnose sarcopenia. This approach can help to establish novel therapies to improve quality of life, exercise capacity, and possibly survival. Chronic heart failure and diabetes are closely linked with each other, although the subgroup of patients with chronic heart failure and type 2 diabetes has received only little systemic research endeavour so far. Insulin is also a powerful anti-lipolytic hormone, thus adipocyte insulin resistance may be indicated by a smaller or missing decrease of free fatty acid release during insulin stimulation. In this cohort, we could confirm changes in lipolytic response to insulin in insulin-resistant patients with heart failure by demonstrating a reduced reduction of free fatty acids with insulin infusion. This finding not only established the presence of insulin-resistance but also demonstrates that increased levels of free fatty acids may be detrimental for cardiac function. Several pathways were analysed in SICA-HF and have been shown to play important roles in heart failure with or without comorbidities. We have identified new biomarkers (C-terminal agrin fragment for identifying patients with skeletal muscle wasting) that may be helpful in clinical settings. Additional potential biomarkers are currently under investigation. We have established, evaluated and formalized a 'panel' or 'system' of criteria allowing the estimation of the regenerative potential of a given patient's own stem cells, for chronic heart failure patients with or without co-morbidities (obesity and/or diabetes mellitus). We found that genotype GG and allele G of apolipoprotein C III gene polymorphism rs2854116, which is associated with disturbances of lipid metabolism, occur two times more frequently in patients with diabetic heart failure than in patients with heart failure without diabetes. We thus suggest that biomarkers and genetic targets identified in SICA-HF should become the target of the subsequent focused studies. In the future, these targets may find some use in the clinical setting.

1.2 Summary description of project context and objectives:

Objective 1: To characterize the prevalence, incidence, persistence, and phenotype of obesity, cachexia, and type 2 diabetes in patients with heart failure

All partners of SICA-HF recruited more than 1462 patients with chronic heart failure, 199 patients with type 2 diabetes without heart failure, and 173 healthy control subjects. Thus, during the last year, a cumulative 91.4% of the target value was achieved for patients with chronic heart failure, 66.3% of the target value for diabetic controls, and 115.3% and thus over-achieving for healthy subjects. The data of all participating centers were collected and entered into the central database. As of this writing, 1469 of all chronic heart failure patients' visits, 255 of diabetic controls' visits, and 167 healthy controls' visits have been entered into the online database. In total, 171 patients have been reported to be deceased at the time of this writing; the total number of hospitalizations is 2232. As part of SICA-HF, a total of 127 skeletal muscle biopsies and 92 fat tissue biopsies have been collected. These patients will allow insight into the incidence and prevalence of type 2 diabetes, obesity, and cachexia in patients with heart failure. Moreover, we will gain insight into the morbidity and mortality of these patients. For all patients, diabetics, and healthy controls, demographic data were recorded, and they completed the EuroHeart Failure Survey Questionnaire and the EQ5D (to

provide symptom profile and quality of life data) and have the following parameters measured; height, weight (recorded using 50g scales), waist and hip circumference, blood pressure (sitting and standing), a 12-lead resting ECG and echocardiogram and 6-minute corridor walk test. They had blood taken for routine biochemistry and haematology; including glucose, lipid profile, glycosolated haemoglobin (HbA1c), hsCRP and NTproBNP (and aliquots stored for plasma bio-markers and DNA). The whole database includes baseline data and follow up data. Additionally outcomes for all patients was followed by record linkage with hospital records for hospitalisation (and reasons) and death.

Objective 2: To describe patterns of exercise capacity and cardiorespiratory reflex control

We have created standardised protocols for cardiopulmonary exercise testing and for cardiopulmonary reflex testing. Patients with assessments of these parameters were followed-up for 28-30 months and re-assessed after 4-6 months, 16-18 months, and at annual intervals thereafter. The aim was to establish exercise capacity, data on heart rate and blood pressure control and baroreflex and chemoreflex sensitivity. Participating centers performed cardiopulmonary exercise testing in 681 of 1462 patients (46.6%) of all patients with chronic heart failure enrolled into SICA-HF, and this value has been already adjusted for those excluded for not reaching the cutoff value for the respiratory exchange ratio of >1.00 . In total, we performed cardiopulmonary exercise capacity 1464 times at baseline and follow-up visits in patients with heart failure. The corresponding number for healthy controls is 153, that for diabetic controls is 18. With regards to baroreflex and chemoreflex sensitivity, we assessed values at 499 visits of patients with heart failure, 54 visits of patients with diabetes, and 29 visits of healthy controls. These data will be integrated with other clinical and preclinical analyses and help in prognosis estimation and analysis of the clinical course. Additionally analyses from cohort of patients in WP5, showed that decreased exercise capacity were strongly associated with iron deficiency and anaemia. Exercise capacity decreased with iron deficiency and more worsened in patients with additionally anaemia. The influence of both comorbidities on exercise capacity opened new insights into the complexity of heart failure.

Objective 3: To analyse body composition and its changes over time in patients with heart failure and type 2 diabetes, obesity, or cachexia.

We aimed to investigate body composition and body composition alterations in patients with chronic heart failure in the context of assessments of the presence of type 2 diabetes, obesity, and cachexia. Body composition analysis using dual energy X-ray absorptiometry (DEXA) was entered into the central database for 987 visits of heart failure patients, for 20 diabetic controls, and for 124 healthy controls. The corresponding values for bioimpedance analysis are 499, 54, and 29. We determined the effects of muscle wasting as assessed using DEXA on the patients' exercise capacity and found 19.5% of patients to be affected by skeletal muscle wasting that has a significant clinical impact on several clinical parameters. These results have been accepted for publication by the European Heart Journal and have been published early in 2013. The study entitled "Muscle wasting in patients with chronic heart failure: results from the studies investigating co-comorbidities aggravating heart failure (SICA-HF)" investigates the prevalence and clinical consequences of muscle wasting fulfilling the criteria of sarcopenia in patients with chronic heart failure. To assess the prevalence and clinical impact of reductions in the skeletal muscle mass of patients with chronic heart failure we prospectively enrolled 200 patients with chronic heart failure. The appendicular skeletal muscle mass was assessed by DEXA. We analysed the muscle strength in arms and legs, and all patients underwent a 6-min walk test, a 4-m walk test, and spiroergometry testing. Muscle wasting was defined as the appendicular muscle mass $\leq 2SD$ below the mean of a healthy reference group of adults aged 18–40 years, as suggested for the diagnosis of muscle wasting in healthy ageing (sarcopenia). Muscle wasting was detected in 39 (19.5%) subjects. Patients with muscle wasting had significantly lower values for handgrip and quadriceps strength as well as lower total peak oxygen consumption (peakVO₂, 1173 ± 433 vs. 1622 ± 456 mL/min), lower exercise time (7.7 ± 3.8 vs. 10.22 ± 3.0 min, both $P < 0.001$), and lower left ventricular ejection fraction (LVEF, $P < 0.05$) than patients without. The distance walked during 6 min and the gait speed during the 4-m walk were lower in patients with muscle wasting (both $P < 0.05$). Serum levels of interleukin-6 were significantly elevated in patients with muscle wasting ($P \leq 0.01$). Logistic regression showed muscle wasting to be independently associated with reduced peak VO₂ adjusted for age, body mass index, LVEF, distance-walked during 6 min, and several co-morbidities (odds ratio 5.19, $P < 0.02$). Muscle wasting is a frequent co-morbidity among patients with chronic heart failure. Patients with muscle wasting present with reduced exercise capacity and muscle strength, and advanced disease.

Objective 4: To investigate the incidence and prevalence of sleep disordered breathing and its impact on the clinical severity in patients with heart failure

Like patients with muscle wasting, those with sleep disordered breathing present with more severe stages of heart failure, i.e. more severe left ventricular dysfunction, worse exercise capacity, and a higher number of co-morbidities. A total of 229 polygraphy assessments for the detection of sleep disordered breathing from patients with chronic heart failure have been entered into online database, 2 for diabetic controls, and 28 for healthy subjects. Patients were evaluated using a standardized questionnaire, an ambulatory sleep-apnoea-screening device, and a 24-hour ECG-monitoring recorder with the measurement of peripheral oxygen saturation. Sleep apnea syndrome (SAS) is an increasingly recognized co-morbidity in patients with heart failure. The aim of our first analysis was to determine exercise capacity and the effects of body composition in patients with sleep disordered breathing. Therefore we included 83 patients according to their Apnea/ Hypopnea Index (AHI). We divided a total of 83 out-patients with stable chronic heart failure the patients according to AHI into four subgroups: group A: AHI <5, n=20 (24.1%), group B: AHI 5-15, n=16 (19.3%), group C: AHI 15-30, n=31 (37.3%), group D: AHI >30, n=16 (19.3%). Up to 76% of patients have signs of SDB. PG can provide only limited information. PSG is required for more detailed analysis. On average, patients with signs of SDB are "more sick", i.e. higher degree of CKD, more severe LV dysfunction, worse exercise capacity. This preliminary analysis shows that values of AHIs as indicators of sleep apnea syndrome are not associated with muscle wasting. Functional parameters like 6-minute-walk are inversely proportional to AHI. Further and larger studies are recommended for better understanding of possible changes in the body composition of patients with sleep apnea syndrome.

Objective 5: To establish the impact of impaired vascular reactivity on impaired skeletal muscle metabolic and functional capacity including its underlying mechanisms

We have analyzed free fatty acids in a subset of patients of who underwent metabolic testing. Patients received an insulin injection and the decrease of blood glucose over 15 minutes is indicative of insulin sensitivity or insulin resistance. Based on the results, patients were divided in 2 groups and cardiac and metabolic parameters were compared. Insulin is also a powerful anti-lipolytic hormone, thus adipocyte insulin resistance may be indicated by a smaller or missing decrease of free fatty acid release during insulin stimulation. In this cohort, we could confirm changes in lipolytic response to insulin in insulin-resistant patients with heart failure by demonstrating a reduced reduction of free fatty acids with insulin infusion. That finding not only established the presence of insulin-resistance but also demonstrates that increased levels of free fatty acids may be detrimental for heart function.

We also performed the ultrastructural analysis of skeletal muscle samples by immunofluorescence and Confocal microscopy analysis. We stained the OCT-embedded cryosections with Laminin and with slow myosin heavy chain (MHC) antibodies. Laminin stains the perimeter of myofibers and slow-MHC stains only myofiber expressing this MHC isoform, which is associated with an oxidative metabolism. Thanks to the laminin staining we can measure the Cross-Sectional Area (CSA) of myofibers. We are interested in CSA, since CSA tends to decrease in atrophy conditions. We concluded that there might be a problem in measuring CSA since the section could be not perpendicular to myofibers; when the cut is oblique, CSA measurement is overestimated. This technical issue must be taken in account before drawing any conclusion. The orientation of the biopsies should be properly regulated and making several attempts might be difficult due to the small size of the sample. The percentage of MHC slow positive myofibers tells us about the metabolism of the muscle. This is of interest, since cachexia is often associated with shifts between glycolytic and oxidative metabolism. In some cases we could not get reliable images since the biopsy was morphologically in bad conditions.

Objective 6: To describe the interplay and metabolic signaling pathways between adipose tissue, skeletal muscle, the bone marrow and the heart in patients with heart failure and type 2 diabetes, obesity, and cachexia

During the project, the Russian partners analyzed genetic polymorphisms associated with ischemic heart disease and heart failure, lipid and fat metabolism, and diabetes mellitus. We found that heart failure associated with diabetes mellitus is characterized by a lower frequency of so called protective genotypes polymorphisms the following genes: genotype TT of TGFb1 gene (TGFb1 869 C>T), genotype AA allele A MMP-9 gene (MMP9 855 A>G), anti-inflammatory genotype CC of IL-6 gene (IL-6 -174 G>C). These genotypes and polymorphisms could be candidates

for the role of genetic biomarkers of unfavorable prognosis in heart failure-associated metabolic disorders. We also found that genotype TT allele T of polymorphisms TCF7L2 gene rs 12253372 is associated with lower insulin increase after glucose administration and can occur more rarely in patients with heart failure and diabetes mellitus than in patients with heart failure without diabetes mellitus or in healthy subjects. Genotype AA of polymorphisms (C-482T) rs2854117 of apolipoprotein C3 gene, is associated with high risk of diabetes mellitus development occurs three times more frequently in patients with heart failure with diabetes mellitus than in patients with heart failure or healthy subjects. The genotype TT of FTO T>A (IVS1) gene polymorphisms, which associated with normal body mass, occurs 2,5 times rarely in patients with heart failure and diabetes mellitus than in healthy subjects. Thus results suggested that these polymorphisms could be candidates for biomarkers of poor prognosis in heart failure aggravated by metabolic disorders. Studying the correlation between gene polymorphisms and biochemical biomarkers we have found that polymorphisms of beta 2-adrenoreceptor gene significantly correlated with NT-proBNP level in patients with heart failure. The reduction in the number of circulating progenitor cells (immunophenotype CD34 +, CD133 dim, CD45-) of less than 300 cells per million white blood cells in patients with chronic heart failure of ischemic aetiology, combined with type 2 diabetes had a negative impact on the course of heart failure, and especially the survival rates and can serve as a clear predictor of cardiovascular mortality. Further cardiomyocytes from patients with heart failure and with heart failure associated with diabetes mellitus were remodelled. In the first case, the preservation of sarcoplasmic reticulum (SR) function contributed to the preservation of the contractile cardiomyocyte reserve. In case of preservation of SR function, development of diabetes mellitus in the presence of heart failure was associated with higher SERCA2a level than in heart failure alone. Oxidative phosphorylation prevailed in the cardiomyocytes of patients with heart failure associated with diabetes mellitus. Development of heart failure alone and diabetes mellitus alone in the experimental animal models led to a decrease in the expression of Ca²⁺-ATPase and ryanodine receptors and deterioration of the glycolysis, Krebs cycle, and oxidative phosphorylation. Moreover, the study of the NO-dependant signaling pathway in the realization of cytokines' effects on the aggregation activity of platelets showed that proinflammatory cytokines tumour necrosis factor and interleukin-1-beta cause significant decrease of the collagen-induced aggregation of the isolated platelets. NO-synthase inhibitor L-NMMA does not influence on the platelet aggregation in the group of patients with heart failure and metabolic disturbances, in contrast to the group of healthy volunteers, which may be caused by the deteriorated expression and activity of the NO-synthase. Activity of NADPH-oxidase generating reactive oxygen species is increased in platelets of patients with heart failure and metabolic comorbidities, which leads to the increase of aggregation. Reactive oxygen species, generated by the extra-platelet enzyme system of xanthine and xanthine-oxidase, decrease platelets' activity in healthy volunteers and increase aggregation in patients with heart failure and metabolic disturbances. According to our results insulin effects on collagen-induced aggregation are realized with participation of the cGMP-dependent-pathways, involving platelets NO-synthase and NO-dependent mechanisms. Metabolic disturbances in patients with heart failure probably lead to the malfunction of cGMP-dependent pathways in platelets, which may be manifested in the increase of platelet aggregation. Study of the cAMP-dependent intracellular signaling system in the insulin-mediated regulation of platelets' aggregational activity showed that addition of IBMX and forskolin to the isolated platelets leads to the significant decrease of the collagen-induced aggregation in the group of patients as well as in the group of healthy volunteers. Absence of differences in the aggregation parameters of the isolated platelets in the groups of patients and health volunteers can prove that metabolic disturbances didn't lead to the change of functioning of the system adenylate cyclase/cAMP in platelets of patients with heart failure and metabolic disturbances, unlike disturbances in the cGMP-dependent signaling pathways functioning. We have performed the first direct comparison of 2 MSC populations established from the same donor. We have demonstrated that at early passages (P2-P3 or up to 14–15 in vitro population doublings), BM- and Ad-derived MSC cultures are comparable in such important characteristics as proliferation rate, clonogenicity and differentiation potential but differ significantly in abundance of CD146 fraction within the sample and in levels of VeGF, SDF-1, MCP1 and tGFβ1 secretion. We have also demonstrated that BM-MSC enter senescence after P3–4, while most F-MSC did not show senescence features up to P6–8. Together, these data demonstrate that specific properties of MSC from different sources should be always taken into account when developing and optimizing the specific protocols for MSC expansion and evaluation for each particular clinical application. Since MSC possess not only plasticity (thus allowing cell replacement) but also prominent secretion features, we aimed investigating variations in secretion properties of MSC established from patients with heart failure and co-morbidities (obesity and/or diabetes). We hypothesized that metabolic alterations associated with the latter will be reflected in altered expression of key genes related to angiogenesis, inflammation, and tissue remodeling in patient-derived BMSCs. We found that BMSCs of heart failure patients with lower body mass index have enhanced expression of genes involved in extracellular matrix remodeling. In particular, body mass index<30 was associated with upregulated expression of genes encoding collagen type I, proteases and protease activators (MMP2, MMP14, uPA), and regulatory molecules

(CTGF, ITGβ5, SMAD7, SNAIL1). In contrast, these transcript levels didn't differ significantly between BMSCs from obese heart failure patients and healthy subjects. In the future, these targets may find some use in the clinical setting. We have established, evaluated and formalized a 'panel' or 'system' of criteria allowing the estimation of the regenerative potential of a given patient's own stem cells, for chronic heart failure patients without or with comorbidities (obesity and/or diabetes mellitus).

Management structure and procedures

The Project Coordinator Prof. Stefan Anker ensured the smooth operation of the project and guaranteed that all efforts were focused towards the objectives. He submitted all required progress reports, deliverables, financial statements to the European Commission, and, with the assistance of GABO:mi he was responsible for the proper use of funds and their transfers to participants. The SICA-HF office was established by and based at the Coordinator in Berlin and at GABO:mi in Munich. The Project Office at the Coordinator was concerned with the scientific management and the co-ordination of all research activities. The Project Office at GABO:mi was responsible for administrative, financial and contractual management and the organisational co-ordination of the project activities.

The Project Governing Board was in charge of the political and strategic orientation of the project and acted as the arbitration body. It met once a year unless the interest of the project required intermediate meetings. The Project Coordination Committee consisted of all work package leaders and the Coordinator and was in charge of monitoring all activities towards the objective of the project in order to deliver as promised, in due time and in the budget. The Project Coordination Committee met every three to six months via telco during the funding period.

1.3 Description of the main S&T results/foregrounds of SICA-HF

WP02 – Statistical support

The primary aim of the research proposed here is to provide the statistical support needed in all WPs of SICA-HF. Improving the outcomes of patients with chronic HF who suffer from type 2 diabetes, obesity, and/or cachexia requires robust data from which conclusions can be drawn. We are committed to rigorous statistical analyzes of the data obtained in SICA-HF to derive such conclusions. In order to maximize output of this WP, we aim to achieve our goals by additionally analyzing existing major databases from large-scale clinical trials that recruited patients with chronic HF or patients who were at risk of developing chronic HF. Overall recruitment has been generally excellent. A joint database was created from data received from all members of the SICA-HF including patients' personal information (recruiting center, date of recruitment, patient initials, date of birth, sex, age) and clinical information (aetiology, weight, height, body mass index, medication, echo parameters, body composition parameters, and work-package specific parameters, etc.) A statistical support to elaborate and write the appropriate Statistical Analysis plans and to then help in solving the questions of descriptive and analytical statistics during the whole study period of SICA-HF was offered. All statistical analyses were carried out using either the Statistical Package for the Social Sciences (SPSS) version 15 for Windows (SPSS Incorporated, Chicago, Illinois, USA), StatView 5.0 software for Windows (Abacus Concepts, Berkley, CA), or MedCalc for Windows version 8.2.0.3 (Broekstraat, Mariakerke, Belgium). All continuous data were checked for normal distribution using the Kolmogorov-Smirnoff test. Non-normal distributed data will be treated as such or transformed to achieve normal distribution (e.g. log-transformation). Statistical analysis use Student's paired and unpaired t test and analysis of variance with Fisher's post hoc test to compare differences between groups for normally distributed variables and using Mann Whitney U-test, Wilcoxon-test, and Kruskal-Wallis-test for non-normal distribution variables, as appropriate. Associations between variables were assessed using univariate or multivariate (step-wise, where appropriate) regression analyses. A value of $p < 0.05$ will be considered significant. To compare different predictive values, areas under the curve (AUC) for sensitivity and specificity will be constructed for relevant variables such as novel biomarkers (WP 10, WP 11) in relevant large patient subgroups. The best prognostic cutoff for survival status is defined as the highest product of sensitivity and specificity. To contrast prognostic accuracy, statistical comparison of receiver operating characteristic curves (ROC) will be performed using the method for paired receiver operating curves described by Hanley and McNeil (Hanley and McNeil, 1983). The relationship of baseline variables with survival will be assessed by Cox proportional-hazards analysis (single predictor and multivariable analysis). Hazard ratios and 95% confidence intervals for risk factors and significance level for c^2 (likelihood ratio test) will be given. To estimate the influence of risk factors on survival,

Kaplan-Meier cumulative survival curves will be constructed and compared by the Mantel-Haenszel log-rank test. We have statistically supported papers and posters from SICA-HF.

We statistically support the paper entitled “Muscle wasting in patients with chronic heart failure: results from the studies investigating co-morbidities aggravating heart failure (SICA-HF)” investigates the prevalence and clinical consequences of muscle wasting fulfilling the criteria of sarcopenia in patients with chronic HF. The aim of this analysis was to assess the prevalence and clinical impact of reductions in the skeletal muscle mass of patients with chronic heart failure. The appendicular skeletal muscle mass was assessed by DEXA. Additionally the analysis of muscle strength in arms and legs, and 6-min walk test, and 4-m walk test, and spirometry testing were taken into account. Muscle wasting was defined as the appendicular muscle mass \leq 2SD below the mean of a healthy reference group of adults aged 18–40 years, as suggested for the diagnosis of muscle wasting in healthy ageing (sarcopenia). Muscle wasting was detected in 39 (19.5%) subjects. Data are presented as mean \pm standard deviation (SD) or median with percentiles. For statistical analysis, StatView 5.0 (SAS Institute, Inc., Cary, USA) was used. Serum levels of IL-1b, IL-6, and TNF- α were non-normally distributed and, therefore, log-transformed to achieve a normal distribution. Analysis of variance (ANOVA), Student’s unpaired t-test, Fisher’s exact test, Pearson’s simple regression, and logistic regression were used as appropriate. A two-tailed P-value \leq 0.05 indicates statistical significance. The results have been accepted for publication by the European Heart Journal and have been published early in 2013. We statistically supported the poster shown in Japan during the 6th cachexia conference. We identify 30% of all patients with AHI>15. We use student’s t-test to compare data between patients and healthy controls. Additionally the second analysis was shown with 4 groups of patients regarding their AHI values. For analysing we use ANOVA test to find statistically relevant parameters. We statistically supported the analysed C-terminal agrin fragment (CAF) in a cohort of 200 patients with and without muscle wasting. The agrin breakdown products TotalCAF, CAF110, and CAF22 were evaluated in heart failure patients with muscle wasting compared to heart failure patients without muscle wasting. Data were presented as mean \pm standard deviation (SD) or median with 25th and 75th percentiles. For statistical analysis, StatView 5.0 (SAS Institute, Inc. Cary, California, United States) was used. Serum levels of CAF, high-sensitivity C-reactive protein (hsCRP), alkaline Phosphatase, gamma-Glutamyltransferase (gamma-GT) creatinine, and ferritin were non-normally distributed and therefore log-transformed to achieve a normal distribution. Analysis of variance (ANOVA), Student’s unpaired t-test, Fisher’s exact test, Pearson’s simple regression, and logistic regression were used as appropriate. Additionally, MedCalc for Windows version 11.2.1.0 (Broekstraat, Mariakerke, Belgium) was used to perform receiver operator characteristic (ROC) curve analysis. Areas under the receiver operator characteristic curve (AUCs) were constructed for sensitivity and specificity to compare different predictive values. We identify CAF as a potential marker of skeletal muscle wasting. The best prognostic cutoff for muscle wasting was defined by the highest product of sensitivity and specificity. The method for paired ROC curves described by Hanley and McNeil was used, performing statistical comparison of ROC curves. A value of $p < 0.05$ was considered to indicate statistical significance in all analyses.

WP03 – Recruitment and characterisation

There were three main component studies that formed the mission of WP3.

Component 1 was a multi-centre study involving (originally 8 centres) that aimed to characterize and recruit approximately 150 healthy controls, 300 patients with diabetes but not heart failure and approximately 1600 patients with heart failure with or without co-morbidities. For patients with heart failure, the intention was to recruit consecutive patients as far as possible. The inclusion criteria for heart failure cohort were:

1. A clinical diagnosis of heart failure
2. Objective evidence of cardiac dysfunction as evidenced by at least ONE of the following within the 6 months prior to enrolment:
 - a. left ventricular ejection fraction \leq 40%;
 - b. left atrial dimension $>$ 4.0 cm (or $>$ 2.5 cm/m in height)
 - c. NT-proBNP $>$ 400 pg/ml [$>$ 47.3 pmol/l] (or BNP $>$ 150 pg/ml)
3. Age $>$ 18 years;
4. Willingness to provide informed consent.

For centres measuring amino-terminal pro-brain natriuretic peptide (NT-proBNP) clinically, only those with plasma concentrations exceeding 400ng/L were mandated for further follow-up as this is the group known to be at greatest risk of deterioration and clinical events.

Component 2 was based on follow-up of the large cohort of patients evaluated in Kingston-upon-Hull that was initiated in 1999 and continues to this day. As the cohort is linked to electronic records follow-up in the UK, follow-up is 100% complete both for hospitalizations and deaths.

Component 3 was a single-centre study again based in Kingston-upon-Hull that aimed to screen 4,000 patients with type-2 diabetes using NT-proBNP to identify patients with cardiac dysfunction and to investigate the prognostic utility of NT-proBNP in this population.

Component Study 1 (All Clinical Centres)

Prospective Characterization of Patients with HF and Their Natural History

Overall, final recruitment was excellent despite some unanticipated difficulties and came close to or exceeded the proposed targets, although it took longer than expected. Given the withdrawal of Rostock and the late accession of Zabrze and difficulties in aligning grant timelines between Russia (that had to initiate spending before the clinical protocol and electronic data-base had been finalised) and the EU centres, we feel very satisfied with what has been achieved in this novel international collaboration between Russia and the EU on a medical-scientific project.

Table 1. Number of patients recruited by cohort and centre.

Centre	Cohort 1 – Healthy Controls	Cohort 2 – Heart Failure only	Cohort 3 – Diabetic Controls	Cohort 4 – Heart Failure and Co-morbidities	Total
Almazov Centre	0	35	0	45	80
Charite	49	100	5	171	325
CRC Moscow	22	35	32	38	127
Golnik	4	0	0	50	54
Hull	27	212	187	330	756
Rome	21	41	16	14	92
Tomsk	10	6	12	62	90
Wroclaw	33	34	3	249	319
Zabrze	0	16	0	27	43
Total	166	479	255	986	1886

Table 2. Number of patient visits for heart failure cohorts recorded in the database for each centre, as at 7 April 2014.

Centre	Baseline	Follow up 1	Follow up 2	Follow up 3	Follow up 4
Almazov Centre	80	2			
Charite	271	211	123	43	1
CRC Moscow	73	55			
Golnik	50	19	5	1	1
Hull	542	329	195	49	
Rome	55	39	15	8	1
Tomsk	68	10			
Wroclaw	283	257	209	113	
Zabrze	43				
Total	1465	922	547	214	3

The table above shows the number of follow-up visits for which data has been entered in the data-base so far. Given the delays in recruitment, the substantial mortality and the fact that follow-up was mandated only if NT-proBNP exceeded 400ng/L the completeness of follow-up is considered satisfactory. Many more follow-up visits will have taken place for clinical purposes and for which data may still become available. Follow-up for clinical events is more robust. The lower rate of follow-up in Russian centres again reflects the difficulties in aligning the timing of grant-spend between Russian and EU centres.

Table 3. Events recorded in the database for patients with heart failure, as at 7 April 2014.

Deaths reported	Deaths adjudicated	Hospitalisations reported
136	134	1002

Centres have been asked to supply that last date known to be alive and therefore follow-up is available for 100% of the patients. The median duration of follow-up at this time is 18 months and the annualised mortality is 7% which is in line with expectations.

In summary, a large cohort of patients has been collected, carefully clinically phenotyped and has good quality medium-term follow-up with a large number of morbid events and substantial mortality. Longer follow-up would be most interesting to observe more of the natural history of heart failure. Plasma, serum, urine and DNA have been collected and stored in Berlin, awaiting bio-marker analysis.

Component 2

The Hull LifeLab cohort provides up to a decade of follow-up on >4,500 patients. It demonstrates that the prognosis of patients referred with suspected heart failure, in whom the diagnosis is subsequently refuted, have, in general, an excellent prognosis even if they have co-morbidities such as diabetes mellitus. NT-proBNP provides more accurate prognostic information than echocardiography, indicating that a simpler, less expensive test that could be done by any doctor or nurse is the preferred test when diagnostic uncertainty exists is a superior test. Patients with a given

plasma concentration of NT-proBNP have a similar prognosis regardless of left ventricular ejection fraction. Multi-variable models suggest that age and NT-proBNP are the two strongest predictors of prognosis. Amongst patients with diabetes, NT-proBNP is again the strongest predictor of outcome. Over a follow-up of a decade, amongst patients who did not have diabetes at baseline, the incidence of diabetes mellitus (defined as an HbA1c >6.5% or >7.0%) was 25% or 10% depending on the threshold used. In summary, component two has been highly successful and will produce a wealth of information in the coming years.

Component 3

Although we failed to fully recruit (due to under-resourcing) to the primary care diabetes cohort, a substantial number of patients was enrolled and the prevalence of disease (raised NT-proBNP) was much higher than expected. Accordingly, the scientific value of this cohort is high. It has already been selected as a paper of special interest to both the Heart Failure Association in Europe and the European Society of Cardiology. This shows that NT-proBNP can identify patients with diabetes mellitus who do not have symptoms of heart disease but who have a substantially greater risk of hospitalisation or death. About 70% of patients with long-standing type-2 diabetes mellitus have an NTproBNP <125ng/L and these patients have an excellent prognosis and a low prevalence of micro-vascular disease. NT-proBNP is a simple tool that can be used both as an inclusion criteria to ensure populations at risk are enrolled in studies and as a useful clinical tool for identifying patients at increased risk who need more active therapy.

Results from the Study

A large number of scientific papers have been presented and these now need to be developed into papers. The SICA-HF programme has highlighted the importance of natriuretic peptides as a diagnostic and prognostic tool in this population, the protective effect of obesity (measured by body mass index and in other ways), the prevalence, incidence and modest additional adverse prognostic significance of diabetes mellitus and the key roles of anaemia, COPD and renal dysfunction in this population, all of which are potential therapeutic targets as well as drivers of an adverse prognosis.

Going forward

European partners intend to submit a further bid under call FPC3 – 2015 of the Horizon 2020 Program to follow up on patients already enrolled in SICA-HF in order to conduct further long-term research into Heart Failure and its common Co-morbidities. This research is optimal when applied to a substantial cohort representative of the epidemiological population (ie not patients from clinical trials) from several centres (to allow for differences amongst populations and clinical practice), with granular data and bio-bank that has been followed for a long-period (in order to show the full natural history of disease). Surprisingly, there are no cohorts of patients that fulfil all of these criteria. SICA-HF could be the first, should resources be found to extend it.

WP04 – QoL, exercise testing & reflex research

The preliminary results of the tests performed within WP4 (CPX and reflexes) in Wroclaw as well as in the others participating centres (CPX only) were presented during the Final PGB Meeting in Berlin in November 2013. The patient recruitment in Wroclaw started in March 2010. Until the end of the project 319 patients have been recruited (283 patients with chronic heart failure, 33 control subjects and 2 diabetic controls). All recruited patients were divided into 4 groups: HF only, HF with accompanying co-morbidities (HF&Co-morb), diabetes only and healthy controls. In all studied groups CPX was performed at baseline visit, and additionally CPX was repeated in patients with HF only and those with HF and co-morbidities after approximately 4-6, 16-18 and 28-30 months. The following test were performed in the enrolled patients: (1) the cardiopulmonary exercise test (CPX), (2) assessment of the cardiorespiratory reflex control using heart rate variability indices, baroreflex sensitivity and central chemoreflex sensitivity. We assessed 319 patients at baseline and performed follow-up visits: 251 patients had first follow-up visit (F/U1), 208 had F/U2 and 113 had F/U3. Most of the patients had CPX test done and approximately 40% of patients had cardiorespiratory reflex control assessed. The obtained measures have been inserted in SICA-HF database.

We also performed the reproducibility study regarding the parameters of cardiopulmonary exercise testing. The reproducibility study concerning CPX parameters was performed in a group of 16 patients. The mean difference

between two CPX test was 7 ± 8 days. The coefficient of variation for peakVO₂ was 7%. The CPX data have been collected in other participating centres (Charite, HULL, Almazov, Rome and Zabrze), and further details are provided in SICA-HF database. All the participating centers performed CPX tests in 681 patients at baseline visit, 406 at F/U1, 252 at F/U2 and 81 at F/U3, respectively. The preliminary analyses of performed tests (CPX and reflexes) were presented during the Final PGB Meeting in Berlin in November 2013.

Cardiopulmonary exercise testing:

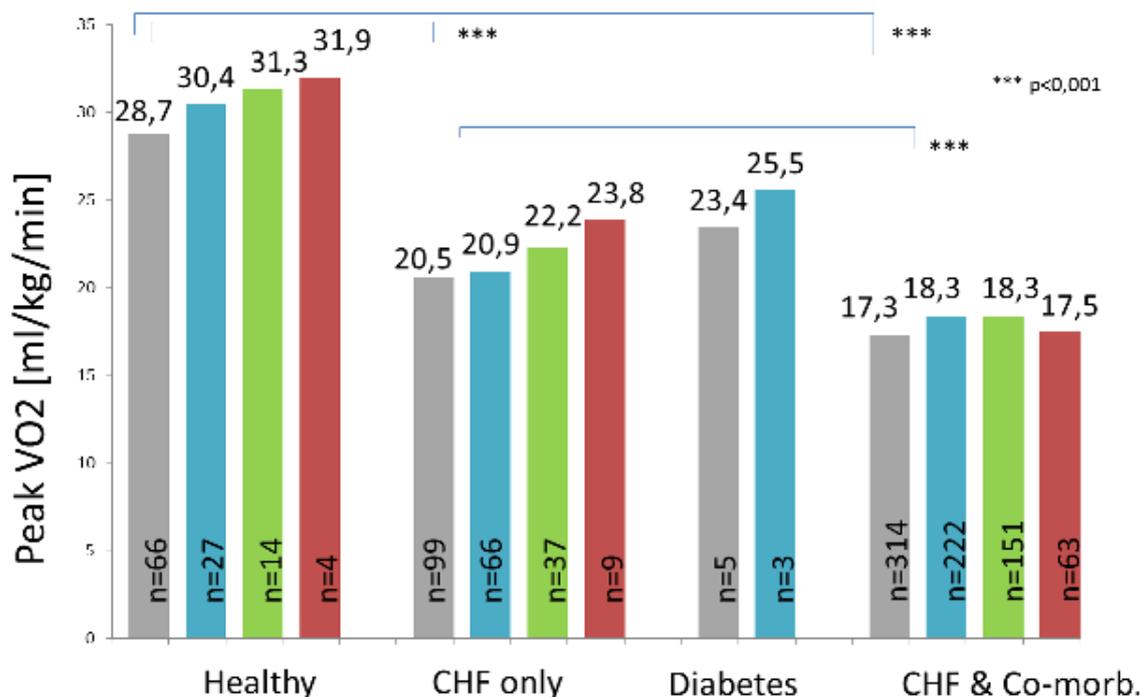
The CPX data have been collected in other participating centres (Charite, HULL, Almazov, Rome and Zabrze), and further details are provided in SICA-HF database. The preliminary analyses of performed tests (CPX and reflexes) were presented during the Final PGB Meeting in Berlin in November 2013. All the participating centers performed CPX tests in 681 patients at baseline visit, 406 at F/U1, 252 at F/U2 and 81 at F/U3.

As shown in the diagram below, peak VO₂ assessed at baseline visit was significantly lower in both groups of HF patients (HF only and HF with co-morbidities) than in healthy subjects. Exercise intolerance was also significantly lower in patients with HF and co-morbidities than in patients suffering from HF only. While comparing 3 consecutive visits peak VO₂ tended to increase in HF-only group (but was not statistically significant), while within the group of HF&Co-morb. there were no significant differences in peak VO₂ between visits.

In the multivariate regression model the following correlations were statistically significant:

a) baseline Peak VO₂ correlated with: Age (B= -0.25; p<0.001), Sex (B= -0.17; p<0.01), BMI (B= -0.20; p<0.01), KCCQ (B= -0.21; p<0.001), Creatinine (B= -0.15; p<0.01), Uric Acid (B= 0.12; p<0.05), NT-proBNP (B= -0.16; p<0.01).

b) baseline VE-VCO₂ correlated with: Weight (B= -0.13; p<0.05), KCCQ (B= -0.18; p<0.01), EF (B= -0.21; p<0.01), Creatinine (B= 0.17; p<0.01), CAD (B= 0.14; p<0.05).



Survival analysis:

Prognostic value of the cardiopulmonary exercise test (CPX) in chronic heart failure with / without diabetes type II was addressed.

During mean follow-up for survivors was 31 ± 8 months (from baseline visit to the end of the study) 47 patients died and 2 patients had heart transplantation. The endpoint was defined as death from any reason or heart transplantation. 1-year survival rate for the whole population was 95.2%. Cox proportional hazard analysis revealed peakVO₂ (expressed as % of predicted but not absolute value) was significant predictor of survival (HR 0.96 , CI 0.92 – 0.99, p=.01). The other significant predictors were VE-VCO₂ slope (HR 1.04 , CI 1.00 – 1.08, p=.04) and the time of exercise (HR 0.97, CI 0.95 – 0.99, p=.01).

In 16-24 months from baseline CPX test in 21% of patients peakVO₂ decreased of 15% and was stable or increased in 97 (79%). Based on change in peakVO₂ over time, patients were divided in 2 groups: Group 1 (true fall defined as peak VO₂ decreased $\geq 15\%$) and Group 2 (peak VO₂ decreased less than 15% or increased). There was significant difference in 1-year survival rate between aforementioned groups: 78,3% and 98,7% respectively (p=0,046).

During mean follow-up 12 ± 7 months (after V3) 11 patients died. For the whole population 1-year survival rate was 91,4 %. The comparison between survivors and non-survivors is shown in the table (Table 6). There were no significant differences in time-related changes in CPX parameters between those groups except from change in peak systolic blood pressure (Δ peakSBP). PeakSBP at reevaluation was significantly lower in patients who died.

Conclusions:

Although single estimates of peak VO₂ (expressed in % of predicted), VE-VCO₂ and the time of exercise had significant prognostic importance, when monitoring changes over time only change in peakSBP was a significant predictor of death. No other time-related change in CPX parameters was significantly related with outcome.

Cardiorespiratory reflex control:

Until the end of the project, in Wroclaw the cardiorespiratory reflex control assessment was performed in 159 patients at the baseline (V1) visit, in 121 patients at the V2, in 88 patients at the V3 visit, in 33 patients at the V4.

HF-Only was characterized by reduced mean RR interval (as compared to both, Healthy Control and HF&Co-morb.) and reduced BRS- α HF (as compared to Healthy Control). In the HF&Co-morb. group, the higher values of mean RR interval and High Frequency HRV (as compared to HF-Only) were observed. BRS- α HF was significantly lower in HF&Co-morb. patients as compared to Healthy Control. There were no other significant differences.

Evaluation of the cardiorespiratory reflex control using heart rate variability (HRV) indices (especially time domain parameters), baroreflex sensitivity and central chemoreflex sensitivity has limited prognostic value in chronic heart failure with / without co-morbidities (e.g. diabetes type II). Contrary to that, frequency domain parameters of HRV (especially, power of HRV spectrum within the high frequency band and the ratio of high frequency HRV to low frequency HRV) may be potentially valuable prognostic factors in these patients, as it was shown that these parameters (baseline values or change in values within 16-24 months) were differ between “survivors” and “non-survivors”. In addition, numerous correlations between frequency domain parameters of HRV and clinical factors (e.g. NT-proBNP) were found.

WP05 - Body composition & Test standardisation

We have prepared a protocol/standard operating procedure for body composition analysis using bioimpedance analysis. This standard operating procedure is available to all partners via the millarium website.

The following methodology is used: To perform a test, the subject is asked to lie down; before measurement the subject should have rested for at least 10 minutes. During this time, waist and hip circumference should be assessed. Two disposable electrodes are placed on the right hand and two on the right foot. The crocodile/alligator clips are attached to the exposed tabs on the electrodes. The appropriate data of gender, height, weight, age, waist and hip circumference are keyed into the unit, the enter key is pressed and measurement is performed. During measurement the patient should lie still, breathing should be calm. Results should be immediately printed out with

the Bluetooth handheld printer, and/or should be downloaded to the computer. Using the downloaded data on the analysing computer “body composition report” should be printed out with the QuadScan 4000 Software.

We have prepared a protocol/standard operating procedure for body composition analysis using DEXA scan. This standard operating procedure is available to all partners via the millarium website.

The following methodology is used: The patient is lying in a supine position with light clothing and without shoes. For the purpose of image analysis, correct alignment of the borders of anatomical regions requires (1) inspection of automated placement of regional borders by the software, (2) manual adjustment of borders for anatomical landmarks according to the following criteria, and (3) Recalculation of regional distribution with the new borders – print – filing.

From February 2010 through September 2013, we recruited a total of 328 subjects. We enrolled 277 patients into the prospective heart failure cohort and 51 subjects in the healthy control and diabetes control cohort. Patients were recruited consecutively, with no pre-specified subgroups. The aim of this study was to recruit 50% women in patients and control groups. In our cohort of patients and controls we have 24% women (248 male and 80 female). Approximately 200 patients with heart failure were recruited over the first 2 years of the study. In addition the aim was to recruit 20 diabetic control subjects and 20 healthy controls with similar age and gender distribution. We recruited a total of 5 diabetic controls and 46 healthy controls in baseline recruitment. Until September 2013 when baseline enrolment was closed a total of 24 patients died during follow up. Mean age of heart failure patients with and without co morbidities were 68.5 ± 11.3 years and the reference group of healthy controls and diabetes controls were 63.8 ± 12.0 years.

Our first results have been accepted for publication by the European Heart Journal and have been published early in 2013. The study entitled “Muscle wasting in patients with chronic heart failure: results from the studies investigating co-morbidities aggravating heart failure (SICA-HF)” investigates the prevalence and clinical consequences of muscle wasting fulfilling the criteria of sarcopenia in patients with chronic HF.

To assess the prevalence and clinical impact of reductions in skeletal muscle mass of patients with chronic heart failure we prospectively enrolled from February 2010 until August 2012 a total of 200 patients with chronic HF. The appendicular skeletal muscle mass was assessed by DEXA. We analyzed muscle strength in arms and legs, and all patients underwent a 6-min walk test, a 4-m walk test, and spiroergometry testing. Muscle wasting was defined as the appendicular muscle mass ≤ 2 SD below the mean of a healthy reference group of adults aged 18–40 years, as suggested for the diagnosis of muscle wasting in healthy ageing (sarcopenia). Muscle wasting was detected in 39 (19.5%) subjects. Patients with muscle wasting had significantly lower values for handgrip and quadriceps strength as well as lower total peak oxygen consumption (peakVO_2 , 1173 ± 433 vs. 1622 ± 456 mL/min), lower exercise time (7.7 ± 3.8 vs. 10.22 ± 3.0 min, both $P < 0.001$), and lower left ventricular ejection fraction (LVEF, $P < 0.05$) than patients without. The distance walked during 6 min and the gait speed during the 4-m walk were lower in patients with muscle wasting (both $P < 0.05$). Serum levels of interleukin-6 were significantly elevated in patients with muscle wasting ($P \leq 0.01$). Logistic regression showed muscle wasting to be independently associated with reduced peak VO_2 adjusted for age, body mass index, LVEF, distance-walked during 6 min, and several co-morbidities (odds ratio 5.19, $P < 0.02$). Muscle wasting is a frequent co-morbidity among patients with chronic heart failure. Patients with muscle wasting present with reduced exercise capacity and muscle strength, and advanced disease.

Further results have been accepted for publication by the Journal of the American Medical Directors Association and have been published in 2013. The study entitled “Resting energy expenditure and the effects of muscle wasting in patients with chronic heart failure: results from the Studies Investigating Comorbidities Aggravating Heart Failure (SICA-HF).” investigates the resting energy expenditure and clinical consequences of muscle wasting in patients with chronic heart failure.

Protein catabolism is characteristic of muscle wasting and contributes to resting energy expenditure (REE). Glucagonlike peptide 1 (GLP-1) is linked to REE in healthy individuals. We aimed to evaluate whether REE is elevated in patients with heart failure with muscle wasting, and whether basal GLP-1 levels are linked to REE in heart failure. In this analysis, 166 patients with chronic HF and 27 healthy controls were included. Body composition was measured by DEXA and muscle wasting was defined as appendicular lean mass of at least 2 SDs below values of a healthy young reference group. REE was measured by indirect calorimetry. Thirty-four of 166 patients (mean age 67.4 ± 10.2 years, 77.7% male, NYHA 2.3 ± 0.6) presented with muscle wasting. We found that patients with heart failure and evidence of muscle wasting showed lower REE than those without muscle wasting. REE in controls and

patients with muscle wasting was significantly lower than in patients without muscle wasting (1579 ± 289 and 1532 ± 265 vs 1748 ± 359 kcal/d, $P = 0.018$ and $P = 0.001$, respectively).

REE normalized for fat-free mass (FFM) using the ratio method (REE/FFM) and analysis of covariance was not different ($P = 0.23$ and 0.71 , respectively). Interestingly GLP-1 did not significantly correlate with REE ($P = 0.49$), even not after controlling for FFM using multivariable regression ($P = 0.15$). These findings seem to be attributable to the lower muscle mass, as after adjustment for FFM, the differences in REE were abrogated. This supports the hypothesis that resting energy metabolism of the skeletal muscle is not impaired in patients with stable, optimally treated HF. Furthermore, we have found that GLP-1 levels do not affect REE in patients with heart failure (Tacke et al.).

Related publications:

- Fülster S, Tacke M, Sandek A, Ebner N, Tschöpe C, Doehner W, Anker SD, von Haehling S. Muscle wasting in patients with chronic heart failure: results from the studies investigating co-morbidities aggravating heart failure (SICA-HF). *Eur Heart J*. 2013, 34(7):512-9. doi: 10.1093/eurheartj/ehs381. PMID: 23178647
- Tacke M, Ebner N, Boschmann M, Jarius A, Valentova M, Fülster S, Sandek A, Schomburg L, Anker SD, Doehner W, von Haehling S. Resting energy expenditure and the effects of muscle wasting in patients with chronic heart failure: results from the Studies Investigating Comorbidities Aggravating Heart Failure (SICA-HF). *J Am Med Dir Assoc*. 2013, 14(11):837-41. doi: 10.1016/j.jamda.2013.08.008. PMID: 24094897

WP06 – Sleep studies

From February 2010 until end September 2013 we recruited a total of 328 subjects. We enrolled 277 patients into the prospective heart failure cohort and 51 subjects in the healthy control and diabetes control cohort. Patients were recruited consecutively, with no pre-specified subgroups. The aim of this study was to recruit 50% women in patients and control groups. In our cohort of patients and controls we have 24% women (248 male and 80 female). Approximately 200 patients with heart failure were recruited over the first 2 years of the study. In addition the aim was to recruit 20 diabetic control subjects and 20 healthy controls with similar age and gender distribution. We recruited a total of 5 diabetic controls and 46 healthy controls in baseline recruitment. All patients are being screened for sleep disordered breathing using a standardised questionnaire, an ambulatory sleep-apnoea-screening device, and a 24-hour ECG-monitoring recorder with the measurement of peripheral oxygen saturation.

Of these patients we followed up a total of 273 for the first follow up after 6 month and 182 patients for follow up after 1 year and 117 patients after 2 years and 37 patients after 3 years. Over all a total of 38 patients died during the follow up. We identify 30% of all patients with $AHI > 15$. Most patients showed obstructive sleep apnea comparing central sleep apnea. Obstructive sleep apnea (OSA) is associated with increased prevalence of type 2 diabetes [1] and higher HbA1C-levels in non-diabetic subjects. Numerous studies have investigated the impact of OSA on insulin resistance. The Sleep Heart Health Study, performed in 2656 individuals, showed that OSA severity was associated with insulin resistance after adjustment for obesity [2] and, in a large Swedish population study on females, OSA was independently associated with insulin sensitivity [3]. OSA comprises various pathophysiological triggers, but beside sleep fragmentation, intrathoracic pressure swings and recurrent hypercapnia, it is the OSA unique form of hypoxia in particular, with repetitive short cycles of desaturation followed by rapid re-oxygenation, termed intermittent hypoxia, that plays a pivotal role in the cardiovascular disease process. The pathogenesis is probably multifactorial and our current concept involves sympathetic nervous system overactivity, systemic inflammation and oxidative stress leading to endothelial dysfunction and, possibly, metabolic dysfunction which are the most important pathways. Figure 1 showed potential pathways of OSA [4]

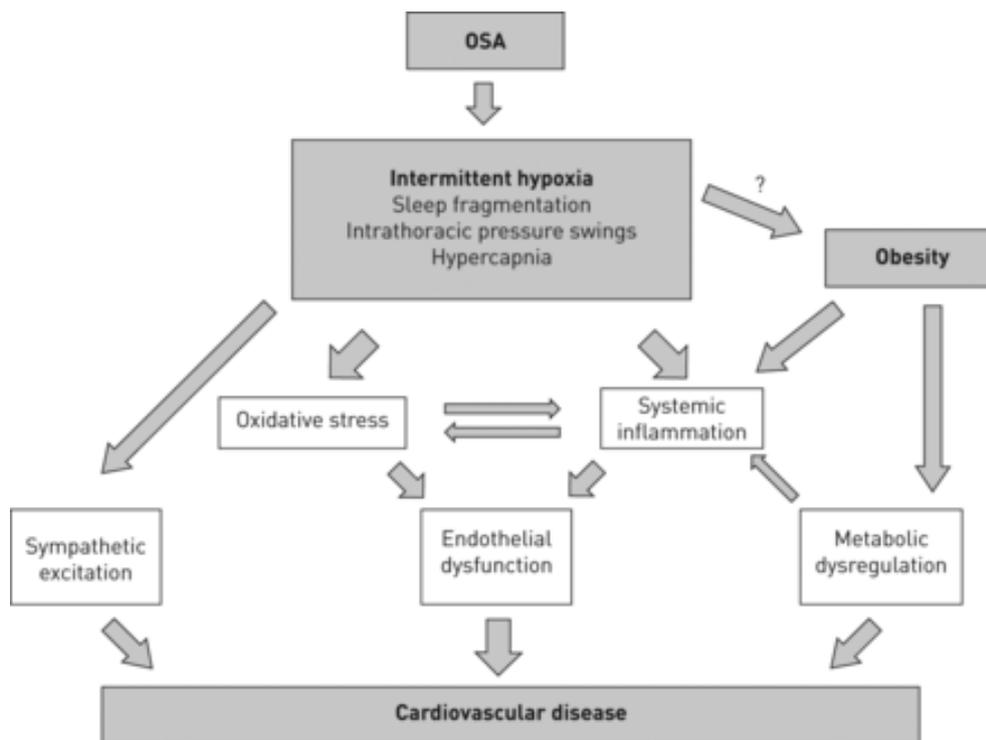


Figure 1: Potential pathways of OSA

- 1) Reichmuth KJ, Austin D, Skatrud JB et al. Association of sleep apnea and type II diabetes: a population-based study. *Am J Respir Crit Care Med* 2005; 172: 1590–1595.
- 2) Punjabi NM, Shahar E, Redline S et al. Sleep-disordered breathing, glucose intolerance, and insulin resistance: the Sleep Heart Health Study. *Am J Epidemiol* 2004; 160: 521–530.
- 3) Theorell-Haglow J, Berne C, Janson C et al. Obstructive sleep apnoea is associated with decreased insulin sensitivity in females. *Eur Respir J* 2008; 31: 1054–1060.
- 4) Lévy P1, Ryan S, Oldenburg O, Parati G. Sleep apnoea and the heart. *Eur Respir Rev* 2013 22(129):333-52. doi: 10.1183/09059180.00004513.

WP07 – Metabolic and vascular studies & biopsies (patients)

Insulin resistance is common in patients with chronic heart failure (HF). Insulin resistance predicted HF incidence independently of established risk factors including diabetes, and is independently associated with impaired prognosis independent of well-established prognostic markers. Beyond mortality, insulin resistance, as a regulating signal of energy utilization, is associated with symptomatic status and exercise capacity in patients with chronic HF. Thus, insulin resistance plays an important role in the pathophysiology in chronic HF with symptomatic and prognostic implications. One of the key clinical features of HF is the reduction in exercise capacity that limits patients' activities of daily life. Such reduction in exercise capacity can be clinically assessed using the peak VO₂ or exercise time assessment and by the measurement of walking distance in the 6-min walk test. Reduced exercise capacity impacts on the patients' quality of life and prognosis. Preserving muscle mass and function can be viewed as pivotal to the maintenance of exercise capacity and, thus, quality of life. Partner WP9, has analyzed free fatty acids in a subset of patients of who underwent metabolic testing. Patients received an insulin injection and the decrease of blood glucose over 15 minutes is indicative of insulin sensitivity or insulin resistance. Based on the results, patients were divided in 2 groups and cardiac and metabolic parameters were compared. Insulin is also a powerful anti-lipolytic hormone, thus adipocyte insulin resistance may be indicated by a smaller or missing decrease of free fatty acid release during insulin stimulation. In this cohort, we could confirm changes in lipolytic response to insulin in insulin-resistant patients with heart failure by demonstrating a reduced reduction of free fatty acids with insulin infusion. That finding not only established the presence of insulin-resistance but also demonstrates that increased levels of free fatty acids may be detrimental for heart function.

WP08 – Cellular research (human muscle)

WP 08's aim was the characterization of functional, ultrastructural and molecular changes in human skeletal muscle tissue in chronic heart failure (CHF) that could potentially be considered as early markers of worsening clinical evolution of the disease. In CHF, functional capacities of the patients are also related to the anatomical and functional status of the skeletal muscle. In response to muscle injury, satellite cells (adult muscle stem cells) are activated for adult muscle maintenance and repair. WP08 also aimed at gaining insight into the cross-talk between muscle, fat and the immune system in these pathological conditions.

As regards the status on recruitment of 50 patients (M08.01), 92 patients have been enrolled. 55 patients with CHF (13 comorbidities), 21 controls and 16 diabetics. 39 CHF patients come back to 2nd visit, 14 to 3rd visit and 2 patients performed 4th visit. 1 patient died and 13 refused to come back (long distance from hospital or other). All CHF patients have been contacted during the last six months and events occurred have been reported on database.

With respect to collection of biopsies, we have collected 35 biopsies; 23 from CHF patients and 12 from healthy subjects to be used as controls). Biopsies were snap frozen in liquid nitrogen. We should also receive from our SICA partners (partner 01, partner 02 and partner 12) involved in WP03 more skeletal muscle biopsies from patients affected by heart failure.

The originally planned genome wide expression analysis has been replaced by the expression study of a smaller gene set, therefore RT-PCR confirmation was not necessary and D08.06 was canceled. However we worked on the expression of skeletal muscle genes in condition of CHF and we analyzed them by quantitative real time PCR (Q-RT-PCR). We report here below our conclusions on the biopsies. In order to identify molecular changes occurring in skeletal muscle in condition of CHF, we decided to test the expression of some relevant structural and regulatory genes involved in muscle mass maintenance. For this purpose we extracted RNA by TRIZOL from the 35 skeletal muscle samples obtained from CHF patients and from healthy people. Since RNA is a very labile molecule subjected to ribonuclease degradation, we checked RNA by electrophoresis on an agarose gel. Unfortunately, we obtained good quality RNA only from 23 out of the total 35 biopsies. 18 biopsies come from CHF patients and 6 from controls. For the gene expression analysis, we decided not to perform wide-range microarray analysis but to evaluate the expression study of a smaller gene set by quantitative Q-RT-PCR. For this purpose we processed all the RNA from the 18 CHF and those from the 6 healthy subjects in order to get cDNA by retrotranscription. From each biopsy we got not more than 3-4 micrograms of RNA. 1 microgram is needed for each retrotranscription reaction and 10 genes may be analyzed. Therefore, about 30 genes per biopsy can be analyzed.

We reasoned on which were the most important genes to be analyzed in order to get informations on the effect of CHF on skeletal muscle mass maintenance. We chose genes whose expression might be modulated in conditions of muscle wasting. They are: MHC (Myosin heavy chain) which codes a myofibrillary protein responsible for skeletal muscle contraction. Foxo1, Foxo4, Foxo3a, Atrogin-1 and MURF-1 are early markers and mediators of skeletal muscle atrophy since they mediate the ubiquitination and subsequent proteasomal degradation of some myofibrillary proteins. Actin was chosen as the normalizing gene. LC3 is a marker of autophagy which is involved in muscle atrophy. Myostatin encodes a secreted protein which negatively regulates skeletal muscle growth. Follistatin contrasts the action of myostatin. Smad2 and Smad4 are involved in the myostatin signaling. Pax3, Pax7, Myogenin, MyoD, MCK (muscle creatin kinase), Myf6, Notch1 and Desmin are involved in satellite cell proliferation, differentiation and in muscle regeneration. IκBα protein inhibits NF-κB complexes which are involved in inflammatory responses. Il-6 cytokine and VCam1 cell surface sialoglycoprotein are involved in inflammation. PGC1-α is a transcription factor playing an essential role in metabolic reprogramming.

We tested all the primers chosen to amplify the correspondent genes on our RNAs. We also tested all RNAs for some of these genes. We therefore now know that we have good quality cDNAs and oligos. However, we have a small number of control biopsies. Obtaining biopsies from healthy subjects was a problem for all groups involved in the recruitment. The perspective for the future is to perform the final gene expression analysis once we get a high number of biopsies and also control biopsies in order to have statistically reliable data and a reliable comparison between control and CHF patients. For this purpose we should receive from our SICA partners (partner 01, partner 02 and partner 12) more skeletal muscle biopsies. We have recently asked them to let us know when they will send us the biopsies.

When we handled for the first time the biopsies, we realised that we could not perform some analysis that we had previously planned and that are indicated mainly in Objective 2. In particular, we have tried to isolate primary human myoblasts from the biopsies but we were not able to do it due to small biopsy size. For the same reason, the cross-talk between muscle, adipose tissue and immune system by co-culture of myogenic primary cells with adipocytes as well as with peripheral blood mononuclear cells (PBMC) extracted from the same patients, was impossible to be performed. We had also proposed that Western Blot analysis of biopsies lysate, together with a deeper study of additional markers by Q-RT-PCR, might allow to obtain relevant informations on muscle physiology. However, the material that could be obtained from the biopsies is not enough for both WB and Q-RT-PCR analysis. Therefore, we decided to perform only RT-PCR for gene expression evaluation. Informations on the cross-talk between muscle and adipose tissue might be obtained from the analysis of some genes such as the perilipins. Moreover, Red Oil staining for lipids will be performed on skeletal muscle cryosections.

We also performed the ultrastructural analysis of skeletal muscle samples by immunofluorescence and confocal microscopy analysis. We stained the OCT-embedded cryosections with laminin and with slow myosin heavy chain (slow-MHC) antibodies. Laminin stains the perimeter of myofibers and slow-MHC stains only myofiber expressing this MHC isoform, which is associated with an oxidative metabolism. Thanks to the laminin staining we can measure the Cross-Sectional Area (CSA) of myofibers. We are interested in CSA, since this parameter tends to decrease in atrophy conditions. We concluded that measuring CSA might be tricky since the section might be not perpendicular to myofibers; when the cut is oblique, CSA measurement is overestimated. This technical issue must be taken in account before drawing any conclusion. The orientation of the biopsies should be properly regulated and making several attempts might be difficult due to the small size of the sample. The percentage of MHC slow positive myofibers tells us about the metabolism of the muscle. This is of interest, since cachexia is often associated with shifts between oxidative and glycolytic metabolism. In some cases we could not get reliable images since the biopsy was morphologically in bad conditions.

We had canceled D08.09 (Report of C2C12 studies on signaling pathways) on the basis of the fact that studies concerning the signaling activated *in vitro*, on C2C12 murine muscle cells cultured in conditions occurring in CHF had already been published. However, despite the deletion of D08.09, we have recently performed a study and we report here, in the dissemination file, the published manuscript (Ferraro et al. 2013) in which we used C2C12 cells treated with TNF α to induce myotube atrophy. TNF α is indeed considered one of the possible cause of cachexia in CHF. We observed that trimetazidine, a metabolic modulator normally accepted for the treatment of CHF patients, acts on some signaling pathways activated in the skeletal muscle cell in atrophy conditions.

WP09 – Cellular research (human fat tissue)

Heart failure and adipose tissue dysfunction appear to interact in a bidirectional fashion. Excessive release of lipolytic substances, such as catecholamines, TNF and natriuretic peptides, and increased susceptibility of adipocytes to these stimuli may occur in heart failure. The mechanism may contribute to adipose tissue wasting in cardiac cachexia patients. On the other hand, disordered adipose tissue function may predispose to heart failure through release of negatively inotropic substances. Changes in adipokine and fatty acid secretion patterns may be involved. Adipose tissue dysfunction is associated with insulin resistance and low grade systemic inflammation, which could, both, worsen heart failure. The overall goal of WP09 is a detailed analysis of adipose tissue dysfunction in heart failure, with emphasis on the accompanying role of comorbidities, e.g. cardiac cachexia, type 2 diabetes, and obesity.

Obesity represents an important risk factor for the development of heart failure, thus the effects of weight loss on heart structure and function in obese patients without overt signs and symptoms of heart failure need to be defined. We demonstrated that weight loss by either a hypocaloric diet reduced in carbohydrates (“Low Carb”) or reduced in fat (“Low Fat”) content is able to reverse left ventricular hypertrophy and also myocardial triglyceride content (Haufe S et al. *Hypertension* 2012). Weight loss may thus be a suitable measure to treat obese patients with cardiac hypertrophy in order to prevent the development of heart failure.

One mechanism by which obesity may contribute to the development of heart failure is the secretion of adipokines with detrimental effects. Fatty acid binding protein 4 (FABP4) is such a candidate. FABP4 has been previously shown to be an adipokine with cardiodepressant effects *in vitro*. We have analyzed FABP4 in relationship to heart size and function in a sample of obese patients and utilized cardio-MR data. We found a relationship with longitudinal

fractional shortening and decreased diastolic function, suggestive of the cardiodepressant effect described *in vitro*. We also found a relationship with left ventricular mass (Engeli S et al. *Heart* 2013). Although the relationship is not strong, most likely because we studied rather healthy subjects, these data are promising and will be replicated within the above mentioned SICA-HF patients. We expect to establish FABP4 as link between heart failure and adipose tissue hypertrophy by these measurements.

Another important adipokine is adiponectin. Adiponectin has been shown to be cardioprotective and antiatherosclerotic. Nevertheless, heart failure patients present with high adiponectin levels that seem to have lost their protective effects. In order to better understand the regulation of adiponectin in heart failure, we infused ANP to obtain plasma concentrations similar to those seen in heart failure patients. We found that ANP in a concentration-dependent manner increased circulating adiponectin. These findings suggest that the increase of adiponectin in heart failure patients does not represent a protective mechanisms but might be simply explained by a natriuretic peptide receptor A mediated stimulatory effect on adipocytes (Birkenfeld AL et al. *PLoS One* 2012).

Natriuretic peptides are also important mediators of natriuresis and vasodilation. In heart failure, increased levels of natriuretic peptides reflect cardiac volume overload and are used as diagnostic and prognostic parameters. On the other hand, we have shown that natriuretic peptides have positive effects on insulin sensitivity and lipid oxidation in skeletal muscle cells (Engeli S et al. *J Clin Invest* 2012). Whereas mitochondrial number and mass is not changed, fatty acid oxidation is increased, and genes of mitochondrial fatty acid transport are activated by natriuretic peptides. Overall, maximal oxygen consumption and electron transport capacity is enhanced which may explained increased insulin sensitivity. These results prompt an important question for future studies: if natriuretic peptides have these metabolic effects, than why and how is this effect lost in heart failure patients who present with massively increased natriuretic peptides but also very often with insulin resistance?

When analysing isolated adipocytes from heart failure patients and control patients without heart failure but matched for medication, age, sex, insulin resistance, and BMI, we found that the lipolytic effects (glycerol release into the culture medium) of isoproterenol and ANP was preserved in the heart failure group (Birkenfeld AL et al. *Hypertension* 2011). Moreover, mRNA expression of natriuretic peptide receptors and of adrenergic receptors on adipocytes revealed no differences between heart failure patients and control patients. These data clearly support our hypothesis that cardiac cachexia might be transmitted at least in part via the preserved response against important lipolytic stimuli like catecholamines and natriuretic peptides that are increased in heart failure patients. These data will be substantiated in further analysis in the SICA-HF cohorts.

WP10 – Blood and DNA bank, biomarker research

The Central Blood and DNA Bank is up and running and can be used to investigate and establish novel biomarkers or panels of biomarkers to better characterize heart failure patients with one or more of the aforementioned co-morbidities in terms of their prognosis, their clinical course (e.g., imminent decompensation of heart failure), and the verification of their clinical diagnosis. Candidate biomarkers include MR-proANP, MR-proADM, high sensitivity troponins, and GDF-15, biomarkers of apoptosis, catabolism, sarcopenia, inflammation, catabolic/anabolic imbalance. For the analysis of biomarkers we require different types of samples, depending on the type of assay. In most cases, EDTA plasma samples are required, however, some assays require usage of serum. For example, whilst MR-proANP (immunoluminometric assay) can be assessed from serum or EDTA plasma, MR-proADM (immunoluminometric assay) can only be assessed from plasma samples (EDTA or lithium heparin). The typical volume required for one test is between 20 and 100 microlitres. In most cases, more than one biomarker can be assessed in one sitting. In addition, most assays require a dead volume of 50-100 microlitres. Therefore, samples are stored in 500 microlitre tubes. We have collected more than 3100 serum 3500 EDTA serum samples from European partners in our biobank.

We study candidate molecules that may help in the characterization of patients with heart failure and obesity/cachexia/sarcopenia and/or diabetes type 2 in the clinical setting on both sides of the consortium the European and on the Russian side. One interesting candidate is Agrin and the C-terminal Agrin Fragment (CAF) as a proteolytic breakdown product of agrin. C-terminal agrin fragment seems to be a potential biomarker for identifying sarcopenia in a subgroup of affected individuals. We involved this biomarker in our analysis. We analysed C-terminal agrin fragment (CAF) in a cohort of 200 patients with and without muscle wasting. We found that the agrin breakdown products TotalCAF, CAF110, and CAF22 were evaluated in heart failure patients with muscle wasting

compared to heart failure patients without muscle wasting. Since the assessment of CAF was associated with high sensitivity, CAF may be useful to identify patients with chronic heart failure and muscle wasting, prompting further investigations in these patients [1,2]. We identify CAF as a potential marker of skeletal muscle wasting.

Some studies showed that the growth differentiating factor-15 (GDF-15) plays an important role in the pathways of muscle wasting and cachexia. GDF-15 is a protein belonging to the transforming growth factor-beta superfamily that has a role in regulating inflammatory and apoptotic pathways during disease processes. Therefore we investigated GDF-15 in our analysis. We analysed GDF-15 and potential proteins that may be important in cachexia and sarcopenia. We included 200 patients with chronic HF and 47 healthy controls of similar age.

Table 1: mean Protein levels in patients with chronic HF (n=200) vs. control (n=47).

Mean protein	Patients	Control	Patients vs. controls
GDF15	2.1	1.0	<0.0001
Activin A	0.4	0.3	0.0004
MIP1b	116.3	91.5	0.0011
IP10	1826.4	1274.8	0.0087
MCP1_MCAF	45.7	37.4	0.0342
IFN γ	609.9	761.7	0.0418
IL4	14.2	16.9	0.0429
IL1b	11.0	13.9	0.0501
IL5	12.3	15.2	0.0745
PDGFbb	586.4	738.4	0.0772

Univariate analysis showed GDF15 is significant associated with HF state in this dataset. HF state as dependent variable and GDF15 (and others) as independent variable were fit in logistic regression model

1) Steinbeck L, Ebner N, Valentova M, Sandek A, Bekfani T, Doehner W, Anker SD, von Haehling S. C-terminal Agrin Fragment as a novel diagnostic marker for muscle wasting in patients with chronic heart failure: Results from the Studies Investigating Co-morbidities Aggravating Heart Failure. *J Cachexia Sarcopenia Muscle* 2014, 5(1) , Abstract 1-32

2) Ebner N, Steinbeck L, Doehner W, Anker SD, von Haehling S. Highlights from the 7th Cachexia Conference: Muscle wasting pathophysiological detection and novel treatment strategies. *J Cachexia Sarcopenia Muscle*. 2014 5(1): 27–34

WP11 – Blood and DNA bank, biomarker research, gene identification

During the project, the Russian partners created the bank of biological samples of full blood, plasma and serum samples from 265 patients (583 serum, 601 plasma, 297 full blood).

Blood samples from 108 patients recruited in CRC, Moscow (22 - control group, 30 - heart failure (HF), 24 heart failure in combination with diabetes (HF+DM) and 32 - type 2 diabetes without HF) were analyzed for genetic polymorphisms associated with ischemic heart disease and HF, lipid and fat metabolism, diabetes mellitus. Aetiology of systolic CHF was ischaemic cardiomyopathy (17 genes and 22 polymorphisms in accordance with the previously described polymorphisms list).

We have found that HF associated with DM type 2 is characterized by lower frequency of so cold protective genotypes polymorphisms the following genes: genotype TT of TGFb1 gene (TGFb1 869 C>T), genotype AA и allele A MMP-9 gene (MMP9 855 A>G), anti-inflammatory genotype CC of IL-6 gene (IL-6 -174 G>C). These genotypes and polymorphisms could be candidates for the role of genetic biomarkers of unfavorable prognosis HF associated with metabolic disorders. We also have found that genotype TT и аллель T of polymorphisms TCF7L2 gene rs 12253372 is associated with lower insulin increase after glucose administration and occur more rarely in

patients with HF+DM2 than in patients with HF without DM and in healthy subjects. Genotype AA of polymorphisms (C-482T) rs2854117 of apolipoprotein C3 gene, which associated with high risk of DM type 2 development occurs three times more frequently in patients with HF+DM2 than in patients with HF or healthy subjects. It was also found that genotype AA and allele A of leptine receptor gene polymorphism rs1137101, which associated with lower level of lipidemia, occurs more rarely in patients with HF+DM2 than in patients with HF. The genotype TT of FTO T>A (IVS1) gene polymorphisms, which associated with normal body mass, occurs 2,5 times rarely in patients with HF +DM2 than in healthy subjects. At the same times genotype AA, which associated with obesity, occurs two times more frequently in patients with HF+DM2 than in healthy subjects. As for genotype GG and allele G of apolipoprotein C III gene polymorphism rs2854116, which associated with disturbances of lipid metabolism, it occurs two times more frequently in patients with HF+DM2 than in patients with HF only and healthy subjects. Thus one can suggest that these polymorphisms also could be candidates for biomarkers of poor prognosis in HG aggravated by metabolic disorders. Studying the correlation between gene polymorphisms and biochemical biomarkers we have found that polymorphisms of beta 2-adrenoreceptor gene significantly correlated with NT-proBNP level in patients with HF (HF and HF+DM2 groups). Genotype GG of gene TCF7L2 polymorphisms rs12253372 correlated with blood level of glucose in HF patients and healthy subjects. The data obtained suggest that prognostic value of all these polymorphisms should be investigated in larger population study.

WP12 – Molecular pathways, cell cultures

Progenitor cells functioning under DM/HF metabolic alterations

We didn't find significant differences in phenotypic profiles and growth kinetics of cultured ADSC from patients with ischemic heart disease, diabetes mellitus type 2 and heart failure compared to the control group.

The total length of capillary-like tube of endothelial cells was significantly decreased in the presence of conditioned media from ADSCs of patients with IHD and diabetes mellitus type 2 or heart failure. The effect of secreted factors from ADSCs of the IHD group demonstrated a decreasing tendency toward their angiogenic activity compared to the control group. We presume that decreasing in angiogenic potentials of ADSCs is explained by disturbance in coordinated network of pro- and anti-angiogenic growth factors.

The reduction in the number of circulating progenitor cells (immunophenotype CD34 +, CD133 dim, CD45-) of less than 300 cells per million white blood cells in patients with chronic heart failure of ischemic etiology, combined with type 2 diabetes had a negative impact on the course of heart failure, and especially the survival rates and can serve as a clear predictor of cardiovascular mortality.

We conclude that the elevated number of progenitor cells is a new biomarker of chronic heart failure, while the reduction of their number is a new biomarker of type 2 DM. High glucose does not affect apoptosis, proliferation and migration but depresses angiogenic properties of progenitor cells. Such risk factors as high glucose and oxLDL impair not only differentiated vascular cells but also progenitor and mesenchymal stem cells, as the result of the decrease of reparative processes in organism.

Correlation between functional activity of sarcoplasmic reticulum Ca²⁺ handling proteins, rhythmotropic reaction and myocardium energetic metabolism in failing hearts with and without diabetes

Cardiomyocytes from patients with HF and with HF associated with DM were remodeled either with the preservation of the SR function or with disruption of the SR function. In the first case, the preservation of SR function contributed to the preservation of the contractile cardiomyocyte reserve.

In case of preservation of SR function, development of DM in the presence of HF was associated with higher SERCA2a level than in HF alone.

Oxidative phosphorylation prevailed in the cardiomyocytes of patients with HF associated with DM.

Development of HF alone and DM alone in the experimental animal models led to a decrease in the expression of Ca²⁺-ATPase and ryanodine receptors and deterioration of the glycolysis, Krebs cycle, and oxidative

phosphorylation.

Induction of hyperglycemia at early stages of HF resulted in smaller changes in the expression of Ca²⁺-ATPase and ryanodine receptors and lesser disturbance of glycolysis, Krebs cycle, and oxidative phosphorylation in comparison with monopathologies.

The role of intraplatelet and extraplatelet enzyme systems, generating reactive oxygen species, and NO-, cAMP-, cGMP-dependent signaling systems, mediating insulin and cytokine effects, in regulation of platelet aggregation activity in patients with association of type 2 diabetes and heart failure

The study of the NO-dependant signaling pathway in the realization of cytokines' effects on the aggregation activity of platelets showed that proinflammatory cytokines tumour necrosis factor and interleukin-1-beta cause significant decrease of the collagen-induced aggregation of the isolated platelets. NO-synthase inhibitor L-NMMA doesn't influence on the platelet aggregation in the group of patients with heart failure and metabolic disturbances, in contrast to the group of healthy volunteers, which may be caused by the deteriorated expression and activity of the NO-synthase.

Activity of NADPH-oxidase generating reactive oxygen species is increased in platelets of patients with heart failure and metabolic comorbidities, which leads to the increase of aggregation.

Reactive oxygen species, generated by the extra-platelet enzyme system of xanthine and xanthine-oxidase, decrease platelets' activity in healthy volunteers and increase aggregation in patients with heart failure and metabolic disturbances.

According to our results insulin effects on collagen-induced aggregation are realized with participation of the cGMP-dependent-pathways, involving platelets NO-synthase and NO-dependent mechanisms. Metabolic disturbances in patients with heart failure probably lead to the malfunction of cGMP-dependent pathways in platelets, which may be manifested in the increase of platelet aggregation.

Study of the cAMP-dependent intracellular signaling system in the insulin-mediated regulation of platelets' aggregational activity showed that addition of IBMX and forskolin to the isolated platelets leads to the significant decrease of the collagen-induced aggregation in the group of patients as well as in the group of healthy volunteers. Absence of differences in the aggregation parameters of the isolated platelets in the groups of patients and health volunteers can prove that metabolic disturbances didn't lead to the change of functioning of the system adenylate cyclase/cAMP in platelets of patients with heart failure and metabolic disturbances, unlike disturbances in the cGMP-dependent signaling pathways functioning.

WP13 – Cellular mechanisms and therapy

Through the whole period of the project, a lot of viable information related to the physiology of mesenchymal stem cells (MSC) of bone marrow and adipose tissue was generated. Numerous experiments have compared cell features in normal physiological conditions (healthy controls, standard culture conditions) and altered conditions (heart failure with and without comorbidities, altered culture conditions). In particular, MSC cell differences and similarities were compared comprehensively. We have performed the first direct comparison of 2 MSC populations established from the same donor. We have demonstrated that at early passages (P2-P3 or up to 14–15 in vitro population doublings), BM- and Ad-derived MSC cultures are comparable in such important characteristics as proliferation rate, clonogenicity and differentiation potential but differ significantly in abundance of CD146 fraction within the sample and in levels of VEGF, SDF-1, MCP1 and tGFβ1 secretion. We have also demonstrated that BM-MSC enter senescence after P3–4, while most F-MSC did not show senescence features up to P6–8. Together, these data demonstrate that specific properties of MSC from different sources should be always taken into account when developing and optimizing the specific protocols for MSC expansion and evaluation for each particular clinical application.

Since MSC possess not only plasticity (thus allowing cell replacement) but also prominent secretion features, we aimed investigating variations in secretion properties of MSC established from patients with heart failure and comorbidities (obesity and/or diabetes). We hypothesized that metabolic alterations associated with the latter will be reflected in altered expression of key genes related to angiogenesis, inflammation, and tissue remodeling in patient-derived BMSCs. We found that BMSCs of heart failure patients with lower body mass index have enhanced expression of genes involved in extracellular matrix remodeling. In particular, body mass index < 30 was associated

with upregulated expression of genes encoding collagen type I, proteases and protease activators (MMP2, MMP14, uPA), and regulatory molecules (CTGF, ITGB5, SMAD7, SNAIL1). In contrast, these transcript levels didn't differ significantly between BMSCs from obese heart failure patients and healthy subjects. Comorbidities (including and diabetes) are known to play role in heart failure progression rate and outcome of the disease. We thus suggest that key molecular targets identified in this study should become the target of the subsequent focused studies. In the future, these targets may find some use in the clinical setting.

We have established, evaluated and formalized a 'panel' or 'system' of criteria allowing the estimation of the regenerative potential of a given patient's own stem cells, for chronic heart failure patients without or with comorbidities (obesity and/or diabetes mellitus). A protocol for such analysis was submitted for registration with Russian state authority as a 'Novel Medical Technology', under the title 'Technology of the complex evaluation of the functional properties of the circulating progenitor cells and mesenchymal stem cells of various tissues (including bone marrow and adipose tissue)'. This technology was successfully applied as 'in vitro part' of the clinical trial Intramyocardial Multiple Precision Injection of Bone Marrow Mononuclear Cells in Myocardial Ischemia (IMPI) launched in Almazov Center. Finally, based on the data generated over the course of SICA-HF study, we have further designed the clinical study entitled 'Intramyocardial Multiple Precision Injection of Various Mesenchymal Stem Cell Populations in Myocardial Ischemia' (acronym = IMPI-MS), and prepared essential documents, necessary for the approval of this study by the institutional Research Council and local Ethical Committee. Namely, the documents prepared include: study protocol, study annotation (the leaflet), informed consent form (IC), individual study participant clinical research form (CRF), Ethical Committee form, standard operative procedures (SOPs) for human biospecimen collection, shipment, processing and analysis. IMPI-MS study goal is a comparative evaluation of the efficacy of intramyocardial precision injection autologous bone marrow and adipose tissue mesenchymal stem cells (MSC) in the treatment of myocardial ischemia. The study structure is 'double blind randomized', and its key characteristic is application of NOGA XP Cardiac Navigation System (Cordis, USA) for both myocardial function/kinetics evaluation and multiple precision injection of autologous stem cells expanded in vitro. Study protocol, with all essential auxiliary documents and forms was submitted to the Institutional (AC) Research Council, and became a subject of discussion. Following the latter, a protocol and associated forms were accepted by the Council. It is apparent that data generated through the WP13 of SICA-HF project, transformed into the panel of criteria allowing the estimation of the regenerative potential of a given patient's own stem cells, will be most useful for the prognosis/prediction of response to autologous MSC transplantation: and its wide application should be inculcated into the clinical practice in the nearest future.

WP14 – Vascular research, ex vivo

In accordance with the objectives of the work package 14, we evaluated the number of circulating progenitor cells (CPCs) in patients with HF (heart failure) and combination HF/DM2 (diabetes mellitus type 2) and healthy volunteers by flow-cytometric analysis; determined the functional characteristics and the angiogenic potential of adipose stromal

Cells (ADSCs) in patients with HF and DM2; evaluated the endothelial dysfunction in relation to the number of CPCs in patients with HF with or without DM2; estimated the relationship between CPCs and circulating angiogenic and antiangiogenic, proinflammatory cytokines in patients with HF and DM2.

The study enrolled 108 patients and classified into four groups: 22 - the control group (healthy volunteers), 30 - patients with heart failure (HF), 24 patients with heart failure, in combination with diabetes (HF+DM2) and 32 - patients with DM2 without HF. Aetiology of systolic CHF was ischaemic cardiomyopathy. Informed consent was obtained from all patients for participation in the study, according to a protocol approved by the Committee on Human Investigation at our institution. All participants underwent a standardized examination that included interviews, anthropometry, BP measurements, a fasting blood draw, resting electrocardiogram, echocardiography.

During the project it was shown that, reducing the number of CD 34⁺ AC133⁺ CPCs in the blood of patients with HF in combination with poor glycemic control DM2 could be a candidate biomarker for aggravation of HF.

Also, we evaluated the circulating growth factors (angiogenic FGF, VEGF, HGF, angiopoietin-1; proinflammatory cytokines IL-6, TNF- α , adipokines). We found increasing in plasma level VEGF, HGF, angiopoietin-1 could be a candidate biomarkers of more severe HF when it is combined with type 2 DM. We don't found significant correlation

between the number of CPCs and endothelial dysfunction parameters. We did not find correlation between the number of circulating progenitor cells and the level of HGF and angiopoetin-1.

Before was reported, that we didn't find significant differences in phenotypic profiles and growth kinetics of cultured ADSCs from patients with ischemic heart disease, DM2 and HF compared to the control group. It has been shown, that ADSCs from patients with cardiovascular diseases and DM2 have impaired angiogenic potential compared to the control group. The total length of capillary-like tube of endothelial cells was significantly decreased in the presence of conditioned media from ADSCs of patients with IHD and DM2 or HF. The effect of secreted factors from ADSCs of the IHD group demonstrated a decreasing tendency toward their angiogenic activity compared to the control group. We presume that decreasing in angiogenic potentials of ADSCs is explained by disturbance in coordinated network of pro- and anti-angiogenic growth factors.

Also, in the during the project was organized monitoring of patients for a period of two to three years.

Outcomes in patients with HF within 2-3 years of follow-up

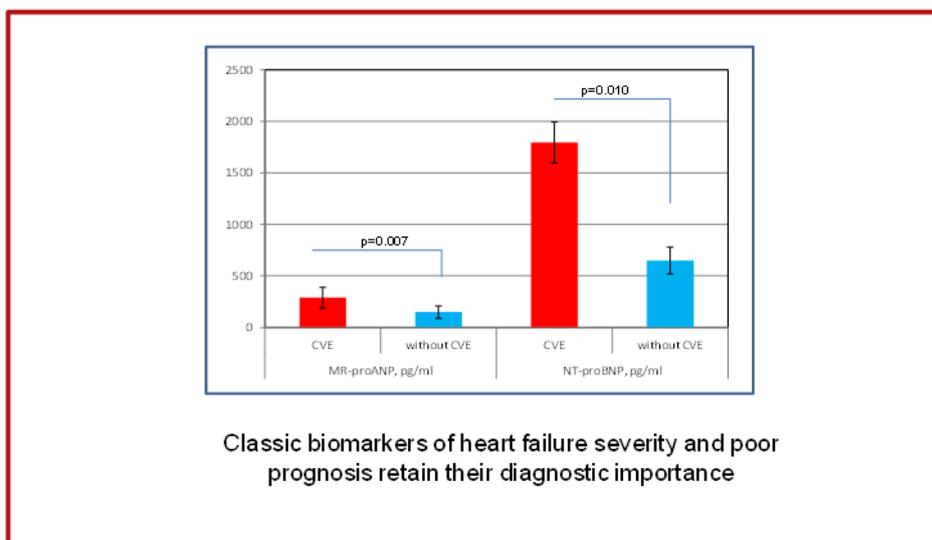


Outcomes (total events=19)			Control(1)	HF(2)	HF+DM2 (3)	DM2(4)	P1-2	P1-3	P2-3	P2-4
CVE	Circulatory decompensation		-	4	2	0	0.01	0.07	0.4	-
	Decomp. IHD	Stent angioplasty	-	2	1	0			0.6	-
		Aorto-coronary by pass surgery	-	1	1	0			0.7	-
Poor glycemc control (decompensation DM2)			-	-	4	2	-	-	-	0.3
Death			1	0	3	0	0.4	0.3	0.08	-

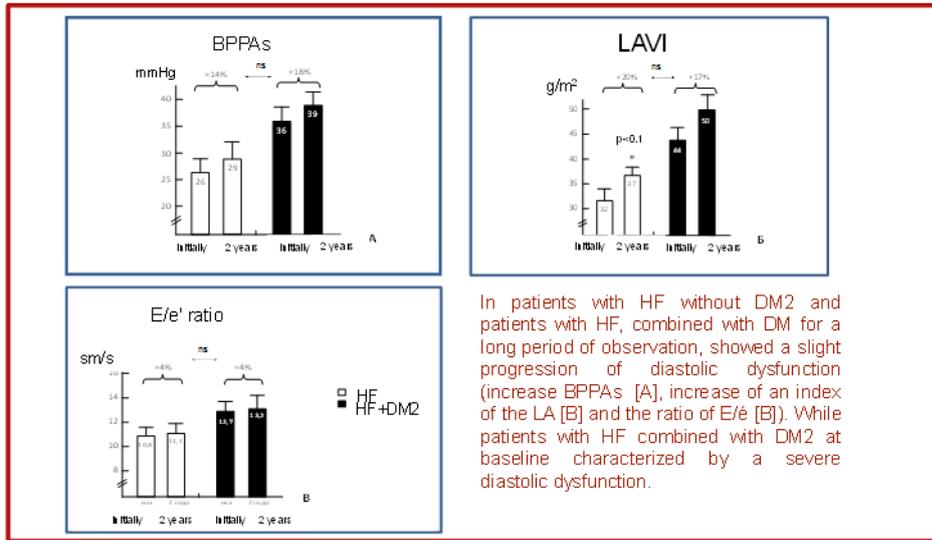
In combined group of patients with chronic heart failure 18 cardiovascular events were recorded, including hospitalization for cardiovascular causes, decompensated diabetes and death.
 3 deaths were registered in the group of patients with chronic heart failure combined with diabetes (2 cases of sudden death, one patient was diagnosed advanced stage of liver cancer and III functional class of heart failure NYHA classification).
 One case of death in control group (causa mortis - prostate cancer).



Adverse CVE associated are with increased level of classic HF biomarkers

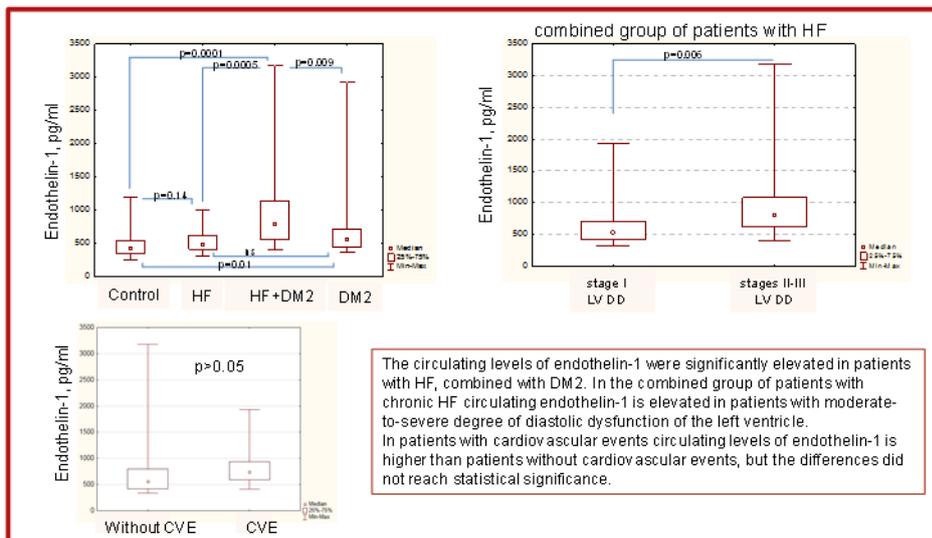


Dynamics of left ventricular diastolic function in patients with chronic systolic HF within 2 years of follow-up



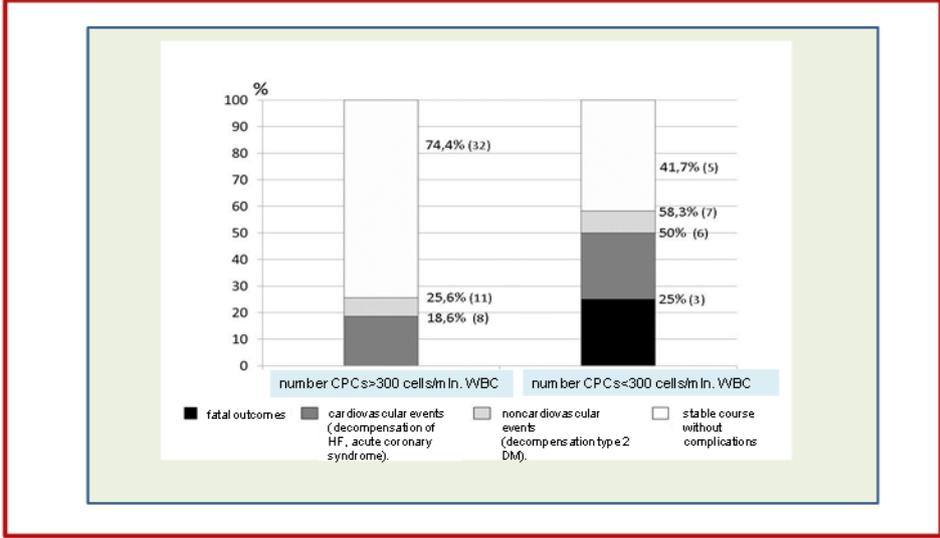
SICA-HF is funded by the European Commission within the 7th Framework Programme

Circulating level of Endothelin-1 in patients with chronic systolic HF correlates with degree of diastolic dysfunction and type 2 diabetes co-morbidity



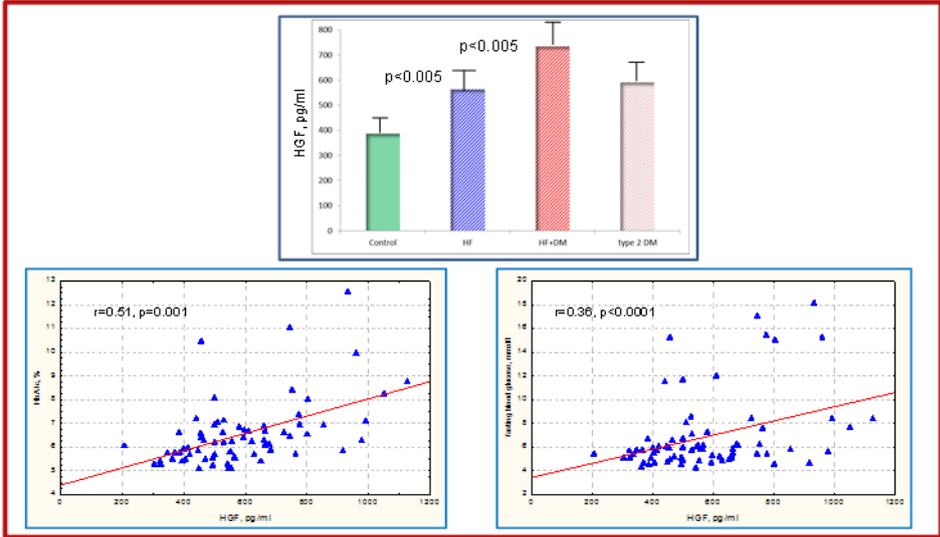
SICA-HF is funded by the European Commission within the 7th Framework Programme

Reduced number of CPCs as a putative predictor of adverse cardio-vascular events



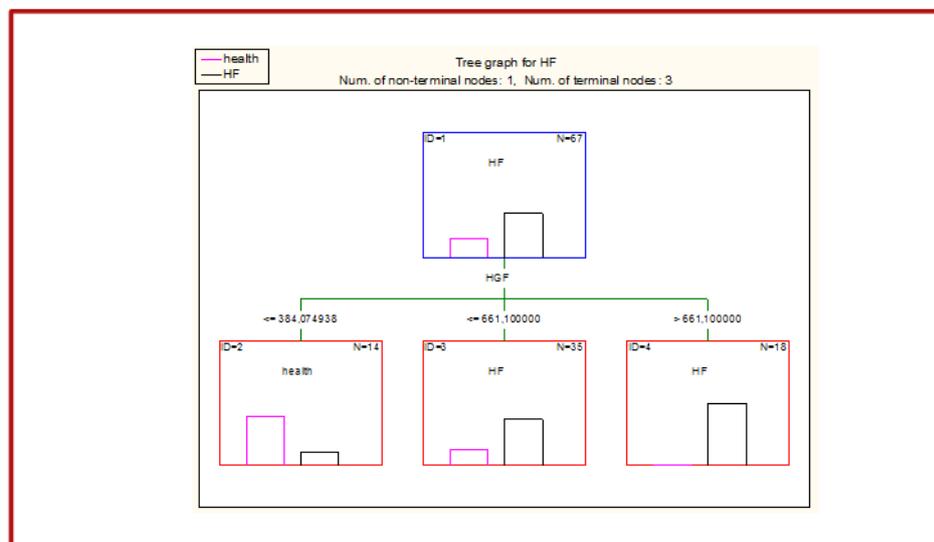
SICA-HF is funded by the European Commission within the 7th Framework Programme

Circulating levels of HGF are elevated in patients with HF/co-morbidity



SICA-HF is funded by the European Commission within the 7th Framework Programme

Classification tree plot for HGF levels and HF presence to determine risk-defining significant HGF elevation



SICA-HF is funded by the European Commission within the 7th Framework Programme

12

Conclusion:

1. The project has been shown that elevated number of circulating progenitor cells is a new biomarker of chronic heart failure. Circulating progenitor cells counts are reduced in DM2 and HF+DM2 patients. Reduced number of circulating progenitor cells is a new biomarker of DM2. Reduced number of CPCs as a putative predictor of adverse cardio-vascular events.
2. In patients with HF as well as with DM2 the level of VEGF in plasma is elevated. Secretion of VEGF by ADSCs of these patients is also higher. These data suggest that HF and DM2 are the result of ischemia of tissues.
3. Circulating levels of endothelin-1 may be a potential biomarker of left ventricular diastolic dysfunction.
4. Circulating levels of HGF may be a marker of chronic heart failure, to be verified by prospective studies. These data may indicate new molecular and cellular mechanisms of heart failure.

WP15 – Academic coordination RUSSIA

During the work, between the consortium partners the interaction was carried out (between countries and within the consortium) as the work of collaboration, both within the individual Work Package (WP) and on the project as a whole.

The 2nd PGB (Project Governing Board) Meeting of SICA-HF took place in St. Petersburg on 3-4 October 2010. The aim of this meeting was to update each other on the ongoing tasks in each Work Package and to discuss about open questions. Also the next steps for the upcoming months have been defined. Necessary information on the project has been exchanged between all project partners. The results of various aspects of mining logistic project, inclusion in the study participants, the technology of biological sample's collection and processing, engineering survey of the research participants, and other topics were presented and discussed at the 2nd PGB. The program of work meeting was included reports and discussion. Ethical conclusions and set the course of the research participants for each project partner; study of cardiopulmonary exercise testing (CPX) and reflex; the status of the Blood and DNA bank; optimized protocols for in vitro model of metabolic disorders; metabolic and vascular studies & biopsies; cellular research, including human muscle and fat tissue; the study of biological cellular mechanisms, technology survey standardization of study participants, work with the database, interaction within the consortium, including international, and interaction with the EC. Certain items on the agenda of the work meeting were held in the format

of the reports focused discussions and a round table discussion, and possible to achieve significant progress in debugging the interaction between partners on many important issues critical to the progress of the project as a whole.

The 3rd PGB (Project Governing Board) Meeting of SICA-HF took place in Rome, Italy on 9-10 October 2011. The participants from all Russian and European partners have attended. The aim of this meeting was to update each other on the ongoing tasks in each workpackage and to discuss about open questions. Also the next steps for the upcoming months have been defined. Concerning the meeting organisation GABO:mi is responsible for the following tasks:

- 1) Preparation of the meeting (finding the date, preparation of the agenda and the overall aim of the meeting, choosing the hotel (including negotiation of the price), online registration for all participants, meeting documents, helpdesk for all questions and queries around the meeting)
- 2) Execution of the meeting (meeting management at the meeting venue), time management, choosing restaurants and catering for lunches and dinners)
- 3) Follow-up (settlement of invoices, drafting the minutes, monitoring of the “next steps”, providing the presentations and documents via the project website, evaluating the meeting via a web-based online surveys)

The workshop on muscle and fat biopsy methodology was held at the Charité, Campus Virchow Klinikum in Berlin, from 27th to 29th November 2011. It was planned and scheduled following the discussions at this year’s annual SICA-HF PGB Meeting in Rome in October. The aim was to coordinate the international project partners on methodology and processing on muscle and fat tissue specimen. Studies on tissue biopsies are central aspects in a number of work packages within the SICA-HF Consortium (WP07, WP08, WP09, WP10, WP12, WP13). Teaching and coordination of common standardized operational procedures across all centers contributing to biopsy studies is therefore a crucial task for the consortium to ensure adequate applicability of methods on an international level.

The 4th PGB (Project Governing Board) Meeting of SICA-HF took place in Moscow, Russia in October 2012. The participants from all Russian and European partners have attended. The aim of this meeting was to update each other on the ongoing tasks in each workpackage and to discuss about open questions. Also the next steps for the upcoming months have been defined. Concerning the meeting organisation GABO:mi is responsible for the following tasks:

- Preparation of the meeting (finding the date, preparation of the agenda and the overall aim of the meeting, choosing the hotel (including negotiation of the price), online registration for all participants, meeting documents, helpdesk for all questions and queries around the meeting)
- Execution of the meeting (meeting management at the meeting venue), time management, choosing restaurants and catering for lunches and dinners)
- Follow-up (settlement of invoices, drafting the minutes, monitoring of the “next steps”, providing the presentations and documents via the project website, evaluating the meeting via a web-based online surveys)

During the work, all Consortium partners had processed the sets of documents required by the local/institutional ethical committees, and have launched study participant recruitment. One of the main tasks of interaction between partners during this stage of work was coordination the principles on which carried the inclusion of participants in the study, their clinical, laboratory and instrumental studies, and so on. The parameters of inclusion into study participant recruitment has been agreed. The parameters/values of clinical and laboratory equipment, collection, processing and storage parameters of the biological samples of different types were discussed. For each of these questions during the discussion, consensus was reached.

The European partners have provided the Russian partners of scientific and technical information, technologies of clinical examination and laboratory studies, some of which were adopted by Russian partners to use in their work.

The European partners have been transferred to the Russian partners a unified and validated protocol for cardiopulmonary exercise testing (CPX), a unified and validated protocol for the assessment of cardiorespiratory reflex control (baroreflex and chemoreflex sensitivity), conversion table of test results, the plan of interview of study participants, methods of NAXFIN.

The protocols of collection and storage of biological material, the systems of deidentification and encoding of personal data have been developed, tested and handed over to the Russian partners by the European partners. These protocols were used to obtain clinical information included in the study participants, for subsequent inclusion in single register study participants, formed by the consortium as a whole (both the Russian and European partners together).

Participants from all centers involved attended the workshop including Hull, UK; Moscow (MSU and CRC), St Petersburg, both RUSSIA; Rome, Italy; Zabrze, Poland, Golnik, Slovenia, and from Hannover, Germany. Experienced senior researchers held presentations of muscle (Dr. Sandek, Dr. Krause) and fat biopsy (Dr. Engeli) handling.

WP16 – Training and dissemination

Website: 55.994 page visits, 20.338 visits; 71% new visitors

The project website www.sica-hf.com has been updated regularly. In the period from 01-10-2009 to 31-03-2014 we had 55.994 visits and 20.338 page visits (71 % new visitors). On the following figure you may see from which countries the visitors are (legend 1-5.142 visitors). The website contains information on patient recruitment and the quarterly newsletter. The internal section was updated regularly containing documents and meeting materials.

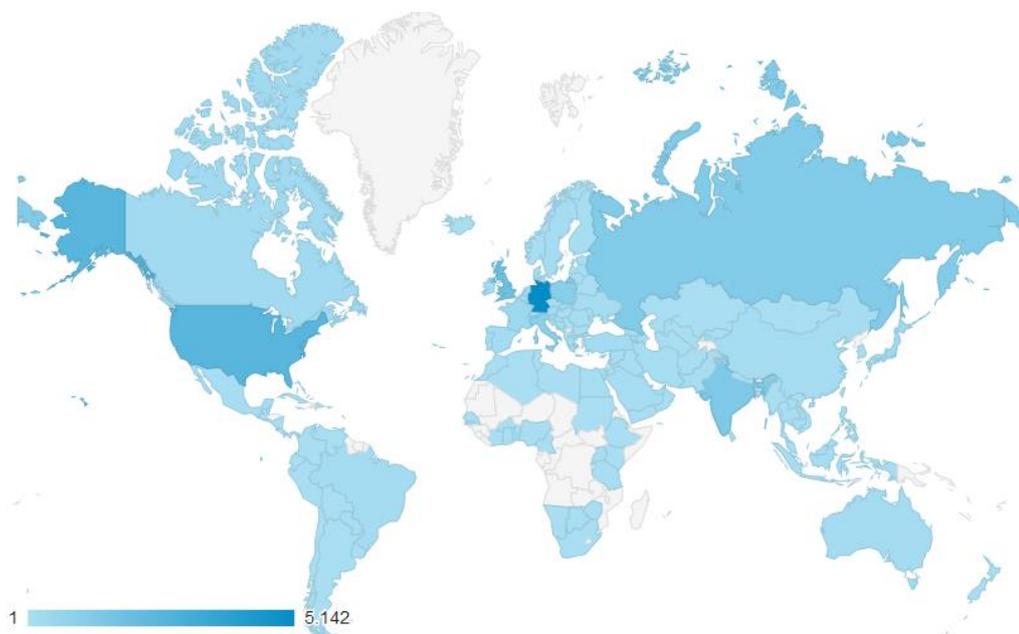


Figure 2 SICA-HF Website Visits per Country

Publications, Posters and Presentations

The SICA-HF consortium disseminated SICA-HF and its result through various channels. A detailed list can be found in ECAS.

Publications (English): 49
 Publications (Russian): 15
 Poster presentations: 35
 Oral presentations: 11
 Press releases: 5
 Interviews: 3

Newsletter:

In the Newsletter, there were presentations of the partner institutes, a distinguished researcher of the consortium, information on project progress, patient recruitment, meetings and relevant publications. In total 10 Newsletter (quarterly) were published and distributed over a mailing list with 54 members.

Project Meetings:

During the course of SICA-HF, there were 5 face-to-face Progress Govering Board Meetings with the whole project consortium. Next to the face-to-face meetings, many telephone conferences were held every 3-6 month, according to needs.

Type of meeting	Date	Venue
Kick-off Meeting	05.-06.10.2009	Berlin, Germany
1 st PCC Telephone Conference	25.01.2010	Telephone Conference
2 nd PCC Telephone Conference	25.03.2010	Telephone Conference
3 rd PCC Telephone Conference	16.06.2010	Telephone Conference
2 nd PGB Meeting	03.-04.10.2010	St. Petersburg, Russia
4 th PCC Telephone Conference	26.04.2011	Telephone Conference
3 rd PGB Meeting	09.-10.10.2011	Rome, Italy
5 th PCC Telephone Conference	16.05.2012	Telephone Conference
6 th PCC Telephone Conference	02.07.2012	Telephone Conference
4 th PGB Meeting/PCC Meeting	13-15 October 2012	Moscow
Meeting on Financial Issues and planned amendment (Coordinator and GABO:mi)	29.11.2012	Berlin
7 th PCC Telephone Conference	18.01.2013	Telephone Conference
8 th PCC Telephone Conference	29.04.2013	Telephone Conference
9 th PCC Telephone Conference	09.09.2013	Telephone Conference

5 th PGB Meeting/PCC Meeting	7-9 November 2013	Berlin
Telco on Preparation of final reporting	02.04.2014	Telephone Conference

For test standardisation SOPs for biopsies, for Parameter of ploygraphy for diagnosing central sleep apnea, for echocardiography, for cardiopulmonary exercise (CPX) was send to all partners and loaded to millarium system. We organised biopsies workshop for training fat and muscle biopsies. During the workshop we organised and show 8 fat and muscle biopsies. During the workshop we discuss and created SOPs for biopsies.

1.4 The potential impact

Socio-economic impact and the wider societal implications of the project

Contribution to Community and social objectives

Heart failure affects more than 14 million individuals in the European Union, and numbers have continued to increase in recent years. The course of heart failure embraces serious implications for patients' quality of life and prognosis. Co-morbidities of heart failure have recently received increasing research endeavour, particularly with regard to anemia, iron deficiency chronic kidney disease, chronic obstructive pulmonary disease, diabetes mellitus, muscle wasting, and cachexia.

Main dissemination activities and exploitation of results

Body composition

Our first results have been accepted for publication by the European Heart Journal and have been published early in 2013. The study entitled "Muscle wasting in patients with chronic heart failure: results from the studies investigating co-morbidities aggravating heart failure (SICA-HF)" investigates the prevalence and clinical consequences of muscle wasting fulfilling the criteria of sarcopenia in patients with chronic HF. To assess the prevalence and clinical impact of reductions in the skeletal muscle mass of patients with chronic heart failure (HF) we prospectively enrolled 200 patients with chronic HF. The appendicular skeletal muscle mass was assessed by DEXA. We analyzed the muscle strength in arms and legs, and all patients underwent a 6-min walk test, a 4-m walk test, and spiroergometry testing. Muscle wasting was defined as the appendicular muscle mass \leq 2SD below the mean of a healthy reference group of adults aged 18–40 years, as suggested for the diagnosis of muscle wasting in healthy ageing (sarcopenia). Muscle wasting was detected in 39 (19.5%) subjects. Patients with muscle wasting had significantly lower values for handgrip and quadriceps strength as well as lower total peak oxygen consumption (peakVO₂, 1173±433 vs. 1622±456 mL/min), lower exercise time (7.7±3.8 vs. 10.22±3.0 min, both $P < 0.001$), and lower left ventricular ejection fraction (LVEF, $P < 0.05$) than patients without. The distance walked during 6 min and the gait speed during the 4-m walk were lower in patients with muscle wasting (both $P < 0.05$). Serum levels of interleukin-6 were significantly elevated in patients with muscle wasting ($P \leq 0.01$). Logistic regression showed muscle wasting to be independently associated with reduced peak VO₂ adjusted for age, body mass index, LVEF, distance-walked during 6 min, and several co-morbidities (odds ratio 5.19, $P < 0.02$). Muscle wasting is a frequent co-morbidity among patients with chronic HF. Patients with muscle wasting present with reduced exercise capacity and muscle strength, and advanced disease. Further results have been accepted for publication by the Journal of the American Medical Directors Association and have been published in 2013. The study entitled "Resting energy expenditure and the effects of muscle wasting in patients with chronic heart failure: results from the Studies Investigating Comorbidities Aggravating Heart Failure (SICA-HF)." investigates the resting energy expenditure and clinical consequences of muscle wasting in patients with chronic HF. Protein catabolism is characteristic of muscle wasting and contributes to resting energy expenditure (REE). Glucagonlike peptide 1 (GLP-1) is linked to REE in healthy individuals. We aimed to evaluate whether REE is elevated in patients with HF with muscle wasting, and whether basal GLP-1 levels are linked to REE in HF. In this analysis 166 patients with chronic HF and 27 healthy controls were included. Body composition was measured by DEXA and muscle wasting was defined as appendicular lean mass of at least 2 SDs below values of a healthy young reference group. REE was measured by indirect calorimetry. Thirty-four of 166 patients (mean age 67.4±10.2 years, 77.7% male, NYHA 2.3 ±0.6) presented with muscle wasting. We found that patients with HF and evidence of muscle wasting showed lower REE than those without muscle wasting. REE in controls and patients with muscle wasting was significantly lower than in patients without muscle wasting (1579 ±289 and 1532±265 vs 1748± 359 kcal/d, $P = 0.018$ and $P = 0.001$, respectively). REE normalized for fat-free mass (FFM) using the ratio method (REE/FFM) and analysis of covariance was not different ($P = 0.23$ and 0.71 , respectively). Interestingly GLP-1 did not significantly correlate with REE ($P = 0.49$), even not after controlling for FFM using multivariable regression ($P = 0.15$). These findings seem to be attributable to the lower muscle mass, as after adjustment for FFM, the differences in REE were abrogated. This supports the hypothesis that resting energy metabolism of the skeletal muscle is not impaired in patients with stable, optimally treated HF. Furthermore, we have found that GLP-1 levels do not affect REE in patients with HF. (Tacke et al.)

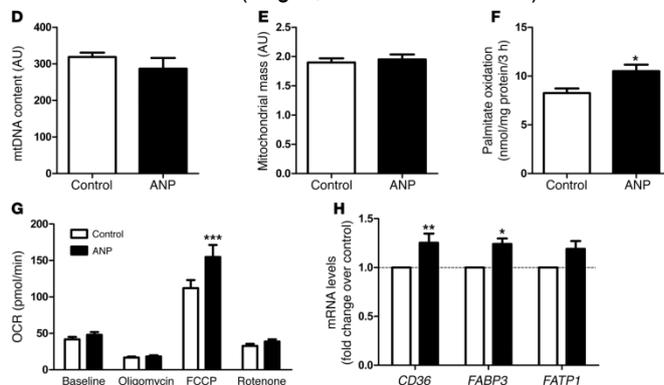
Sleep studies

All partners of SICA-HF recruited more than 1462 patients with chronic heart failure, 199 patients with type 2 diabetes without heart failure, and 173 healthy control subjects. Thus, during the last year, a cumulative 91.4% of the target value was achieved for patients with chronic heart failure, 66.3% of the target value for diabetic controls, and 115.3% and thus over-achieving for healthy subjects. The data of all participating centers were collected and entered into the central database. As of this writing, 1469 of all chronic heart failure patients' visits, 255 of diabetic controls' visits, and 167 healthy controls' visits have been entered into the online database. Participating centers performed cardiopulmonary exercise testing in 681 of 1462 patients (46.6%) of all patients with chronic heart failure enrolled into SICA-HF, and this value has been already adjusted for those excluded for not reaching the cutoff value for the respiratory exchange ratio of >1.00 . Body composition analysis using dual energy X-ray absorptiometry (DEXA) was entered into the central database for 987 visits of heart failure patients, for 20 diabetic controls, and for 124 healthy controls. The corresponding values for bioimpedance analysis are 499, 54, and 29. We determined the effects of muscle wasting as assessed using DEXA on the patients' exercise capacity and found 19.5% of patients to be affected by skeletal muscle wasting that has a significant clinical impact on several clinical parameters. Like patients with muscle wasting, those with sleep disordered breathing present with more severe stages of heart failure, i.e. more severe left ventricular dysfunction, worse exercise capacity, and a higher number of co-morbidities. A total of 229 polygraphy assessments for the detection of sleep disordered breathing from patients with chronic heart failure have been entered into online database, 2 for diabetic controls, and 28 for healthy subjects. In total, 171 patients have been reported to be deceased at the time of this writing; the total number of hospitalizations is 2232. At the Charité in Berlin, approximately 200 patients with heart failure were recruited over the first 2 years of the study. All patients were screened for the presence of sleep-disordered breathing using a standardised questionnaire, an ambulatory sleep-apnoea-screening device with the measurement of peripheral oxygen saturation. Sleep apnea syndrome (SAS) is an increasingly recognized co-morbidity of patients with heart failure. The aim of our first analysis was to determine exercise capacity and the effects of body composition in patients with sleep disordered breathing. Therefore we included 83 patients according to their Apnea/ Hypopnea Index (AHI). We divided a total of 83 out-patients with stable chronic heart failure the patients according to AHI into four subgroups: group A: AHI <5 , $n=20$ (24.1%), group B: AHI 5-15, $n=16$ (19.3%), group C: AHI 15-30, $n=31$ (37.3%), group D: AHI >30 , $n=16$ (19.3%). Up to 76% of patients have signs of SDB. PG can provide only limited information. PSG is required for more detailed analysis. On average, patients with signs of SDB are "more sick", i.e. higher degree of CKD, more severe LV dysfunction, worse exercise capacity. This preliminary analysis shows that values of AHIs as indicators of sleep apnea syndrome are not associated with muscle wasting. Functional parameters like 6-minute-walk are inversely proportional to AHI. Further and larger studies are recommended for better understanding of possible changes in the body composition of patients with sleep apnea syndrome.

Fat biopsies

As part of SICA-HF, a total of 127 skeletal muscle biopsies and 92 fat tissue biopsies have been collected. As part of the basic research endeavour in SICA-HF, we analyzed a number of metabolic signaling pathways such as fatty acid binding protein 4 (FABP4) and its relationship to heart size and function in a sample of obese patients with heart failure (Engeli, Heart 2013). FABP4 has been previously shown to be an adipokine with cardiodepressant effects. The relationship between obesity and heart failure lead us to analyze FABP4 in this sample of obese patients with cardio-MR data. We found a relationship with longitudinal fractional shortening and decreased diastolic function, suggestive of the cardiodepressant effect described in vitro. We also found a relationship with left ventricular mass. Although the relationship is not strong, most likely because we studied rather healthy subjects, these data are promising and will be replicated within the above mentioned SICA-HF patients. We expect to establish FABP4 as link between heart failure and adipose tissue hypertrophy by these measurements. We have analyzed free fatty acids in a subset of patients of who underwent metabolic testing. Patients received an insulin injection and the decrease of blood glucose over 15 minutes is indicative of insulin sensitivity or insulin resistance. Based on the results, patients were divided in 2 groups and cardiac and metabolic parameters were compared. Insulin is also a powerful anti-lipolytic hormone, thus adipocyte insulin resistance may be indicated by a smaller or missing decrease of free fatty acid release during insulin stimulation. In this cohort, we could confirm changes in lipolytic response to insulin in insulin-resistant patients with heart failure by demonstrating a reduced reduction of free fatty acids with insulin infusion. That finding not only established the presence of insulin-resistance but also demonstrates that increased levels of free fatty acids may be detrimental for heart function.

Natriuretic peptides are important mediators of natriuresis and vasodilation. In heart failure, increased levels of natriuretic peptides reflect cardiac volume overload and are used as diagnostic and prognostic parameters. On the other hand, we have shown that natriuretic peptides have positive effects on insulin sensitivity and lipid oxidation in skeletal muscle cells (Engeli, *J Clin Invest* 2012). Whereas mitochondrial number and mass is not changed (D,E),



fatty acid oxidation is increased (F), and genes of mitochondrial fatty acid transport are activated by natriuretic peptides (H). Overall, maximal oxygen consumption and electron transport capacity is enhanced (G). These results prompt an important question for future studies: if natriuretic peptides have these metabolic effects, than why and how is this effect lost in heart failure patients who present with massively increased natriuretic peptides but also very often with insulin resistance.

Muscle biopsies

For the first analysis a total of 92 subjects have been enrolled. Fifty-five patients with chronic heart failure (13 with comorbidities), 21 controls and 16 diabetics. Thirty-nine patients come back to 2nd visit, 14 to 3rd visit and 2 patients performed an additional visit. 1 patient died and 13 refused to come back (long distance from hospital or other causes). All CHF patients have been contacted during the last 12 months and events eventually occurred have been reported on SICA database.

We set up optimal conditions for tissue biopsies handling in order to perform Q-RT-PCR and immunofluorescence analysis of markers of interest. We standardized procedures to carry out skeletal muscle biopsies from different centers and to correctly freeze and store tissues. This aspect turned out to be critical for efficient RNA extraction and analysis through Q-RT-PCR and for immunofluorescence analysis. We extracted good quality RNA and cDNA from 23 biopsies (18 biopsies from CHF patients and 6 from controls). We also tested the primers for Q-RT-PCR of the 23 genes listed above. The perspective for the future is to perform the final gene expression analysis once we get a higher number of biopsies also from healthy subjects, in order to have statistically reliable data and a reliable comparison between control and CHF patients. For this purpose we asked our SICA partners (partner 01, partner 02 and partner 12) involved in WP03 which should provide more skeletal muscle biopsies, necessary to achieve 100% of the tasks. Also our WP partner 11 (CRC) has been contacted but we never receive any answer. We also performed the ultrastructural analysis of skeletal muscle samples by immunofluorescence and Confocal microscopy analysis. We stained the OCT-embedded cryosections with Laminin and with slow myosin heavy chain (MHC) antibodies. Laminin stains the perimeter of myofibers and slow-MHC stains only myofiber expressing this MHC isoform, which is associated with an oxidative metabolism. Thanks to the laminin staining we can measure the Cross-Sectional Area (CSA) of myofibers. We are interested in CSA, since CSA tends to decrease in atrophy conditions. We concluded that there might be a problem in measuring CSA since the section could be not perpendicular to myofibers; when the cut is oblique, CSA measurement is overestimated. This technical issue must be taken in account before drawing any conclusion. The orientation of the biopsies should be properly regulated and making several attempts might be difficult due to the small size of the sample. The percentage of MHC slow positive myofibers tells us about the metabolism of the muscle. This is of interest, since cachexia is often associated with shifts between glycolytic and oxidative metabolism. In some cases we could not get reliable images since the biopsy was morphologically in bad conditions.

Blood and DNA bank, biomarker research

The Central Blood and DNA Bank is up and running and can be used to investigate and establish novel biomarkers or panels of biomarkers to better characterize heart failure patients with one or more of the aforementioned comorbidities in terms of their prognosis, their clinical course (e.g., imminent decompensation of heart failure), and the verification of their clinical diagnosis. Candidate biomarkers include MR-proANP, MR-proADM, high sensitivity

troponins, and GDF-15, biomarkers of apoptosis, catabolism, sarcopenia, inflammation, catabolic/anabolic imbalance. For the analysis of biomarkers we require different types of samples, depending on the type of assay. In most cases, EDTA plasma samples are required, however, some assays require usage of serum. For example, whilst MR-proANP (immunoluminometric assay) can be assessed from serum or EDTA plasma, MR-proADM (immunoluminometric assay) can only be assessed from plasma samples (EDTA or lithium heparin). The typical volume required for one test is between 20 and 100 microlitres. In most cases, more than one biomarker can be assessed in one sitting. In addition, most assays require a dead volume of 50-100 microlitres. Therefore, samples are stored in 500 microlitre tubes. We have collected more than 3100 serum 3500 EDTA serum samples from European partners in our biobank.

We study candidate molecules that may help in the characterization of patients with heart failure and obesity/cachexia/sarcopenia and/or diabetes type 2 in the clinical setting on both sides of the consortium the European and on the Russian side. One interesting candidate is Agrin and the C-terminal Agrin Fragment (CAF) as a proteolytic breakdown product of agrin. C-terminal agrin fragment seems to be a potential biomarker for identifying sarcopenia in a subgroup of affected individuals. We involved this biomarker in our analysis. We analysed C-terminal agrin fragment (CAF) in a cohort of 200 patients with and without muscle wasting. We found that the agrin breakdown products TotalCAF, CAF110, and CAF22 were evaluated in heart failure patients with muscle wasting compared to heart failure patients without muscle wasting. Since the assessment of CAF was associated with high sensitivity, CAF may be useful to identify patients with chronic heart failure and muscle wasting, prompting further investigations in these patients. We identify CAF as a potential marker of skeletal muscle wasting. Some studies showed that the growth differentiating factor-15 (GDF-15) plays an important role in the pathways of muscle wasting and cachexia. GDF-15 is a protein belonging to the transforming growth factor-beta superfamily that has a role in regulating inflammatory and apoptotic pathways during disease processes. Therefore we investigated GDF-15 in our analysis. We analysed GDF-15 and potential proteins that may be important in cachexia and sarcopenia. We included 200 patients with chronic HF and 47 healthy controls of similar age. Univariate analysis showed GDF15 is significant associated with HF state in this dataset. HF state as dependent variable and GDF15 (and others) as independent variable were fit in logistic regression model. There is further need for attractive biomarkers as therapeutic targets.

Blood and DNA bank, gene identification

During the project, the Russian partners created the bank of biological samples of full blood, plasma and serum samples from 265 patients (583 serum, 601 plasma, 297 full blood). Blood samples from 108 patients recruited in CRC, Moscow (22 - control group, 30 - heart failure (HF), 24 heart failure in combination with diabetes (HF+DM) and 32 - type 2 diabetes without HF) were analyzed for genetic polymorphisms associated with ischemic heart disease and HF, lipid and fat metabolism, diabetes mellitus. Aetiology of systolic CHF was ischaemic cardiomyopathy (17 genes and 22 polymorphisms in accordance with the previously described polymorphisms list). We have found that HF associated with DM type 2 is characterized by lower frequency of so cold protective genotypes polymorphisms the following genes: genotype TT of TGFb1 gene (TGFb1 869 C>T), genotype AA allele A MMP-9 gene (MMP9 855 A>G), anti-inflammatory genotype CC of IL-6 gene (IL-6 -174 G>C). These genotypes and polymorphisms could be candidates for the role of genetic biomarkers of unfavorable prognosis HF associated with metabolic disorders. We also have found that genotype TT и allele T of polymorphisms TCF7L2 gene rs 12253372 is associated with lower insulin increase after glucose administration and occur more rarely in patients with HF+DM2 than in patients with HF without DM and in healthy subjects. Genotype AA of polymorphisms (C-482T) rs2854117 of apolipoprotein C3 gene, which associated with high risk of DM type 2 development occurs three times more frequently in patients with HF+DM2 than in patients with HF or healthy subjects. It was also found that genotype AA and allele A of leptine receptor gene polymorphism rs1137101, which associated with lower level of lipidemia, occurs more rarely in patients with HF+DM2 than in patients with HF. The genotype TT of FTO T>A (IVS1) gene polymorphisms, which associated with normal body mass, occurs 2,5 times rarely in patients with HF +DM2 than in healthy subjects. At the same times genotype AA, which associated with obesity, occurs two times more frequently in patients with HF+DM2 than in healthy subjects. As for genotype GG and allele G of apolipoprotein C III gene polymorphism rs2854116, which associated with disturbances of lipid metabolism, it occurs two times more frequently in patients with HF+DM2 than in patients with HF only and healthy subjects. Thus one can suggests that these polymorphisms also could be candidates for biomarkers of poor prognosis in HG aggravated by metabolic disorders. Studying the correlation between gene polymorphisms and biochemical biomarkers we have found that polymorphisms of beta 2-adrenoreceptor gene significantly correlated with NT-proBNP level in patients with HF (HF

and HF+DM2 groups). Genotype GG of gene TCF7L2 polymorphisms rs12253372 correlated with blood level of glucose in HF patients and healthy subjects. The data obtained suggest that prognostic value of all these polymorphisms should be investigated in larger population study.

Molecular pathways, cell cultures

The total length of capillary-like tube of endothelial cells was significantly decreased in the presence of conditioned media from ADSCs of patients with IHD and diabetes mellitus type 2 or heart failure. The effect of secreted factors from ADSCs of the IHD group demonstrated a decreasing tendency toward their angiogenic activity compared to the control group. We presume that decreasing in angiogenic potentials of ADSCs is explained by disturbance in coordinated network of pro- and anti-angiogenic growth factors. The reduction in the number of circulating progenitor cells (immunophenotype CD34 +, CD133 dim, CD45-) of less than 300 cells per million white blood cells in patients with chronic heart failure of ischemic aetiology, combined with type 2 diabetes had a negative impact on the course of heart failure, and especially the survival rates and can serve as a clear predictor of cardiovascular mortality. We conclude that the elevated number of progenitor cells is a new biomarker of chronic heart failure, while the reduction of their number is a new biomarker of type 2 DM. High glucose does not affect apoptosis, proliferation and migration but depresses angiogenic properties of progenitor cells. Such risk factors as high glucose and oxLDL impair not only differentiated vascular cells but also progenitor and mesenchymal stem cells, as the result of the decrease of reparative processes in organism.

Cardiomyocytes from patients with HF and with HF associated with DM were remodelled either with the preservation the SR function or with disruption of the SR function. In the first case, the preservation of SR function contributed to the preservation of the contractile cardiomyocyte reserve. In case of preservation of SR function, development of DM in the presence of HF was associated with higher SERCA2a level than in HF alone. Oxidative phosphorylation prevailed in the cardiomyocytes of patients with HF associated with DM. Development of HF alone and DM alone in the experimental animal models led to a decrease in the expression of Ca²⁺-ATPase and ryanodine receptors and deterioration of the glycolysis, Krebs cycle, and oxidative phosphorylation. Induction of hyperglycemia at early stages of HF resulted in smaller changes in the expression of Ca²⁺-ATPase and ryanodine receptors and lesser disturbance of glycolysis, Krebs cycle, and oxidative phosphorylation in comparison with monopathologies.

The study of the NO-dependant signaling pathway in the realization of cytokines' effects on the aggregation activity of platelets showed that proinflammatory cytokines tumour necrosis factor and interleukin-1-beta cause significant decrease of the collagen-induced aggregation of the isolated platelets. NO-synthase inhibitor L-NMMA doesn't influence on the platelet aggregation in the group of patients with heart failure and metabolic disturbances, in contrast to the group of healthy volunteers, which may be caused by the deteriorated expression and activity of the NO-synthase. Activity of NADPH-oxidase generating reactive oxygen species is increased in platelets of patients with heart failure and metabolic comorbidities, which leads to the increase of aggregation. Reactive oxygen species, generated by the extra-platelet enzyme system of xanthine and xanthine-oxidase, decrease platelets' activity in healthy volunteers and increase aggregation in patients with heart failure and metabolic disturbances. According to our results insulin effects on collagen-induced aggregation are realized with participation of the cGMP-dependent-pathways, involving platelets NO-synthase and NO-dependent mechanisms. Metabolic disturbances in patients with heart failure probably lead to the malfunction of cGMP-dependent pathways in platelets, which may be manifested in the increase of platelet aggregation. Study of the cAMP-dependent intracellular signaling system in the insulin-mediated regulation of platelets' aggregational activity showed that addition of IBMX and forskolin to the isolated platelets leads to the significant decrease of the collagen-induced aggregation in the group of patients as well as in the group of healthy volunteers. Absence of differences in the aggregation parameters of the isolated platelets in the groups of patients and health volunteers can prove that metabolic disturbances didn't lead to the change of functioning of the system adenylate cyclase/cAMP in platelets of patients with heart failure and metabolic disturbances, unlike disturbances in the cGMP-dependent signaling pathways functioning.

Cellular mechanisms and therapy

Through the whole period of the project, a lot of viable information related to the physiology of mesenchymal stem cells (MSC) of bone marrow and adipose tissue was generated. Numerous experiments have compared cell features in normal physiological conditions (healthy controls, standard culture conditions) and altered conditions (heart failure with and without comorbidities, altered culture conditions). In particular, MSC cell differences and similarities were compared comprehensively. We have performed the first direct comparison of 2 MSC populations established from

the same donor. We have demonstrated that at early passages (P2-P3 or up to 14–15 in vitro population doublings), BM- and Ad-derived MSC cultures are comparable in such important characteristics as proliferation rate, clonogenicity and differentiation potential but differ significantly in abundance of CD146 fraction within the sample and in levels of VeGF, SDF-1, MCP1 and tGFβ1 secretion. We have also demonstrated that BM-MSC enter senescence after P3–4, while most F-MSC did not show senescence features up to P6–8. Together, these data demonstrate that specific properties of MSC from different sources should be always taken into account when developing and optimizing the specific protocols for MSC expansion and evaluation for each particular clinical application. Since MSC possess not only plasticity (thus allowing cell replacement) but also prominent secretion features, we aimed investigating variations in secretion properties of MSC established from patients with heart failure and co-morbidities (obesity and/or diabetes). We hypothesized that metabolic alterations associated with the latter will be reflected in altered expression of key genes related to angiogenesis, inflammation, and tissue remodeling in patient-derived BMSCs. We found that BMSCs of heart failure patients with lower body mass index have enhanced expression of genes involved in extracellular matrix remodeling. In particular, body mass index < 30 was associated with upregulated expression of genes encoding collagen type I, proteases and protease activators (MMP2, MMP14, uPA), and regulatory molecules (CTGF, ITGβ5, SMAD7, SNAIL1). In contrast, these transcript levels didn't differ significantly between BMSCs from obese heart failure patients and healthy subjects. Comorbidities (including and diabetes) are known to play role in heart failure progression rate and outcome of the disease. We thus suggest that key molecular targets identified in this study should become the target of the subsequent focused studies. In the future, these targets may find some use in the clinical setting.

We have established, evaluated and formalized a 'panel' or 'system' of criteria allowing the estimation of the regenerative potential of a given patient's own stem cells, for chronic heart failure patients without or with comorbidities (obesity and/or diabetes mellitus). A protocol for such analysis was submitted for registration with Russian state authority as a 'Novel Medical Technology', under the title 'Technology of the complex evaluation of the functional properties of the circulating progenitor cells and mesenchymal stem cells of various tissues (including bone marrow and adipose tissue)'. This technology was successfully applied as 'in vitro part' of the clinical trial Intramyocardial Multiple Precision Injection of Bone Marrow Mononuclear Cells in Myocardial Ischemia (IMPI) launched in Almazov Center. Finally, based on the data generated over the course of SICA-HF study, we have further designed the clinical study entitled 'Intramyocardial Multiple Precision Injection of Various Mesenchymal Stem Cell Populations in Myocardial Ischemia' (acronym = IMPI-MSC), and prepared essential documents, necessary for the approval of this study by the institutional Research Council and local Ethical Committee. Namely, the documents prepared include: study protocol, study annotation (the leaflet), informed consent form (IC), individual study participant clinical research form (CRF), Ethical Committee form, standard operative procedures (SOPs) for human biospecimen collection, shipment, processing and analysis. IMPI-MSC study goal is a comparative evaluation of the efficacy of intramyocardial precision injection autologous bone marrow and adipose tissue mesenchymal stem cells (MSC) in the treatment of myocardial ischemia. The study structure is 'double blind randomized', and its key characteristic is application of NOGA XP Cardiac Navigation System (Cordis, USA) for both myocardial function/kinetics evaluation and multiple precision injection of autologous stem cells expanded in vitro. Study protocol, with all essential auxiliary documents and forms was submitted to the Institutional (AC) Research Council, and became a subject of discussion. Following the latter, a protocol and associated forms were accepted by the Council. It is apparent that data generated through the WP13 of SICA-HF project, transformed into the panel of criteria allowing the estimation of the regenerative potential of a given patient's own stem cells, will be most useful for the prognosis/prediction of response to autologous MSC transplantation: and its wide application should be inculcated into the clinical practice in the nearest future.

Outlook and future research

Several pathways have been shown to play important roles heart failure with or without co-morbidities. We have identified new biomarkers (CAF, GDF-15) that may be useful in clinical settings. Additional potential biomarkers are currently under investigation. We have established, evaluated and formalized a 'panel' or 'system' of criteria allowing the estimation of the regenerative potential of a given patient's own stem cells, for chronic heart failure patients without or with comorbidities (obesity and/or diabetes mellitus). We found that genotype GG and allele G of apolipoprotein C III gene polymorphism rs2854116, which associated with disturbances of lipid metabolism, occurs two times more frequently in patients with HF+DM2 than in patients with HF. We thus suggest that biomarkers and genetic targets identified in SICA-HF should become the target of the subsequent focused studies. In the future, these targets may find some use in the clinical setting.

Section 2 – Use and dissemination of foreground

2.1 Plan for use and dissemination of foreground (including socio-economic impact and target groups for the results of the research)

Section A

List of Scientific Publication

For more information please see the ECAS system.

List of Dissemination Activities

For more information please see the ECAS system.

Section B

No patents, trademarks, registered designs, etc. were applied.

Section 3 – Report on societal implications

For more information please see the ECAS system.