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Part 1. Executive summary

At the beginning of 2010 BIOCLAIMS entered in a practically virgin area in the agrofood sector related with health, that of the research to identify and characterize new biomarkers using nutrigenomics and other technologies with a horizon of its potential use as basis for future health claims made on food in Europe. The big challenge was that no appropriate biomarkers existed for many potential health-promoting effects of foods, thus precluding its substantiation as health claims under the very rigorous scientific context and the enormous change represented by the progressive implementation of EU legislation (Regulation EC Nº 1924/2006). Given the long road (decades for markers or risk factors nowadays accepted by EFSA) that is expected to be covered by a plausible biomarker candidate until its full exploitation, it was considered urgent to start ASAP collaborative research in this topic, which was therefore granted at the forefront of a number of other EU and national public and private ulterior initiatives.

As planned, the project has resulted in the identification and characterization of nutrigenomics-based biomarkers and other biomarkers of health robustness or other health benefits, including relevant mechanistic data supporting its biological plausibility. Various identified biomarkers have been verified in different animal models and/or human studies and a big load of scientific information is now available for its potential application to substantiate beneficial effects of nutrients or bioactive compounds in infants and adults, leading or contributing to potential innovative food health claims.

In particular, some more promising results can be highlighted regarding the problem of obesity and related comorbidities. A first nutrigenomics-based biomarker has been identified (published and patented) in blood cells (BC) and its usefulness is being studied to predict/prevent/treat in early life a potential large proportion of obesities that develop in adults in later life. Also using BC, transcript-based biomarkers of both early priming of metabolic health and adipose tissue expandability sensitive to dietary intervention have been identified. On another hand, circulating levels of specific lysophospholipids could be used as a early biomarker for dyslipidemia; and plasma metabolome focused on acylcarnitines and amino acids could be used as a gender-specific complex biomarker of propensity to obesity and possibly obesity-associated metabolic disorders. Indirect calorimetric analysis (and other parameters) in response to fed/fasting conditions reflects early loses of homeostatic robustness. From another approach, a combination of protein damage biomarkers discovered in urine appears able to detect and discriminate between good health and early decline in metabolic, vascular and renal health in humans. A number of the above and other results generated by the consortium are under review, they will be further assessed, could also be further confirmed and its IP protected in a near future. Between those, preliminary results on early biomarkers of pre-diabetes aggravation, new markers of resistance to low-grade

inflammation associated with obesity, as well as newly described effects of n-3 PUFA on plasma parameters in response to a meal (which could lead to "improved satiety" claim) are going to be further assessed; among other new advances.

Stakeholders consulted have already shown great interest in these results, as reflected in the more recent interaction meetings. From here, the involvement of parts (industry) more directly interested in the development and exploitation of concrete biomarkers or related mechanistic information is ongoing or is expected in the following years on a case by case basis.

Part 2. Summary description of project context and objectives

Robust biomarkers are crucial for assessing the potential effectiveness and benefits of health-promoting food compounds. This is the basis for new and competitive economic and health developments in the food sector as covered by the Regulation (EC) Nº 1924/2006 of the European Parliament and of the Council of 20 December 2006, which established rules aimed at harmonising 'health claims (HC) made on food' at an European level for the first time (1, 2).

From then on, health claims on food labelling, presentation and advertising which states, suggests or implies that a relationship exists between a food or one of its constituents and health, must be clear, concise and based on evidence accepted by the whole scientific community. Beyond an apparent simplicity, the HC framework is considered the more complex piece of legislation ever adopted in Europe, and 9 years later it is still a matter of successive developments and interpretations. From the refined list of 4.637 items representing potential health claims (starting from a preliminary list of more than 44 000 slogans present in the EU marked in 2006) only 260 have been authorized so far (April 2015) having sufficient scientific evidence as judged by EFSA. This balance has been in agreement with the outcome estimated in the BIOCLAIMS proposal, with many of the unfavourable opinions affected by the use of inappropriate biomarkers. In addition, only 13 out of the 260 authorized HC correspond to reduction of disease risk claims, a particular type of biomarkers, which are based so far on just 4 concrete 'risk factors': cholesterol, folic acid, dental plaque and/or pH, and bone mineral density. In successive published guidelines/criteria, other biomarkers and risk factors have been mentioned by the EFSA NDA competent Panel, which correspond to parameters coming from the medical world and that have been beneficed by decades of R+D and clinical practice. About 2000 claims intended for botanical extracts have been put on hold in 2010 by the EC and are still pending for a decision, which still requires more science and, probably, new types of biomarkers and the reassessment of the EU Regulation. The lack of biomarkers is still the main bottleneck for expanding the food-health developments and has become a leading R+D priority topic in both the EC and in most EU member states; consequently, after 2010 several projects on HC have been set up and are running; while in 2009 BIOCLAIMS was the first European consortium applying to a large Collaborative R+D project addressing the search for new biomarkers as the basis for future health claims made on food.

The challenge is that for a number of physiological functions affected by food compounds there are no useful biomarkers described and for other functions there is a need for earlier biomarkers (3, 4). This lack is the main bottleneck for the consolidation and expansion of the health claims-based added values in the food sector. Moreover, instead of relying only on disease-oriented biomarkers used in the biomedical world and making derivative biomarkers for nutrition research out of these ("surrogate end points"), a new focus on biomarkers towards maintenance of physiological function and integrity is needed, both to better understand physiology and to cope with the above mentioned novel EU legislation.

In addition to the traditional approaches and technologies in nutrition and metabolic regulation studies, BIOCLAIMS proposed, at the edge of its proposal, the use of the novel omics technologies, previously experienced by part of the groups in the EU research network NUGO, and in particular the Nutrigenomic approaches. A nutrigenomic-based biomarker is defined as a set of information consisting mainly of quantitative levels of gene expression and/or proteins and/or metabolites that, when appropriately combined, reflects the health status of a given physiologically relevant process. Both transcriptomics and metabolomics have been proved to be very useful in identifying and characterizing new biomarkers for future health claims (see next section).

BIOCLAIMS aimed at:

a) identifying and characterizing nutrigenomic-based, early, robust biomarkers predictive of a healthy metabolic phenotype during ageing and/or facing stressors to homeostasis;

b) comparing and validating these novel, emerging biomarkers against traditional markers; and

c) testing the response of these novel, emerging biomarkers to bioactive food components, in animal and human models.

The physiological significance of selected biomarker candidates was proposed to be assessed at the whole-organism level in different animal models and in humans under both free living and controlled conditions (prospective cohorts and intervention studies) and with known genetic background regarding a pro-inflammatory genotype.

BIOCLAIMS main general objectives were, therefore:

- To develop novel nutrigenomic-based biomarkers for characterizing highly prevalent conditions of impaired homeostatic control;
- To provide scientific evidence on effect of aging and gender on biomarkers of homeostatic control;
- To provide scientific evidence on the effects of early priming on biomarkers of healthy aging;
- To develop biomarkers for mapping the intrinsic effects of bioactive food components present in the normal diet under conditions of homeostatic control covering a continuum ranging from normal to impaired.

In the end, these new knowledge might provide scientific evidence to help support future health claims on food.

The general strategy, as originally proposed, has been be to compare two conditions, one (healthy), that is leading to the maintenance of a given health function for a longer period, and the other (distorted) that is expected (due to pre-imposed genetic or acquired alterations) to lead to a shorter period of health functioning (see the FIGURE). To reveal homeostatic differences in an early stage, before clear health differences are visible, a challenge (stress) is applied. From here, robustness of the system is used as a predictor for later differences in health.

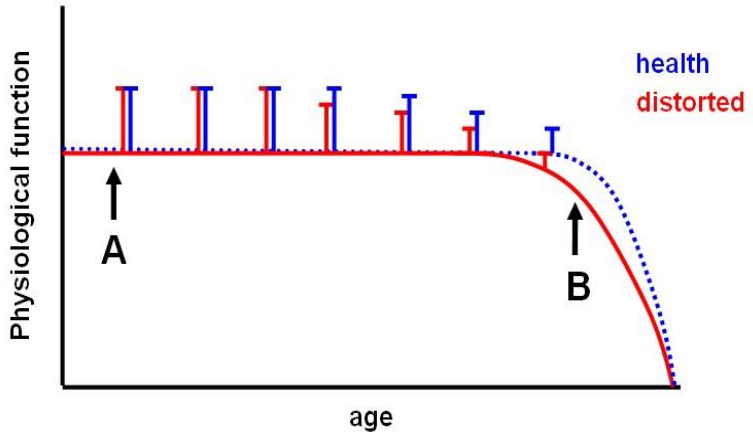

Figure 2 of the BIOCLAIMS proposal. ***Biomarkers of health and of distorted states.*** *Robustness, elasticity and resilience of the mechanisms involved in maintaining homeostasis decrease during age, resulting in loss of physiological function. New nutrigenomic early biomarkers will be identified at point A to be able to predict functional differences (early loss of function when ageing) later on in life (point B). Parameters characterizing biological systems will be identified and compared in early and later stages (A and B in the figure, respectively) to reveal homeostatic differences before clear health differences are visible.*

Metabolic and vascular health and related disorders are core to BIOCLAIMS. This is so because:

a) Metabolic and vascular health is a major health goal. Because of the public health importance of vascular disease and metabolic disorders (obesity, diabetes type 2, hepatosteatosis, etc), the importance of identifying early biomarkers for metabolic and vascular health is manifest, in particular if they can be modified or improved by diet and food bioactives and these effects can be conveyed as health claims according to the EU legislation.

b) (Cardio) vascular disease is a solid endpoint that has established risk factors and biomarkers to which novel biomarkers can be compared.

c) Metabolic and vascular health is well interconnected with the two other major emerging health problems (obesity and insulin resistance; and thus, type 2 diabetes), the blood pressure controlling system, renal function and other related complications in the border of manifest diseases.

d) Metabolic and vascular health can be nutritionally strengthened, and certainly consumers and producers alike have the strong feeling that this is the case.

e) Important gender differences in cardiovascular health have to be considered.

f) There are a number of still unknown interconnections and metabolic signals relating early homeostatic alterations to outcomes related to metabolic and vascular health. For instance, we have realised and contemporary research suggests that the adipocyte-derived hormone leptin may be an important factor linking obesity, the metabolic syndrome, and cardiovascular disorders (5, 6). Increasing the knowledge on these signals and processes, and their mechanisms and response to bioactive substances, is also of great interest.

References Part 2:

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

Most used abbreviations: HFD, high fat diet: InCa, indirect calorimetry; PBMC, peripheral blood mononuclear cells; PUFA, polyunsaturated fatty acids; WAT, white adipose tissue; WP, work package (within the Project)

BIOCLAIMS has developed and extensively characterized a variety of animal models and humans from the points of view of physiology, transcriptomics, lipidomics, metabolomics, and protein and lipoprotein damage analysis **in seeking for biomarkers of health, early disruption of metabolic homeostasis and early priming of metabolic health**. Animal studies (conducted in work packages (WP) 1 and 2) aimed mainly at developing novel biomarkers, while human studies (grouped in WP3) aimed at validating biomarkers identified in animal studies, developing novel biomarkers, and cross-validating these novel candidate biomarkers with established ones in human subjects. **Animal models in BIOCLAIMS** included a variety of nutritional interventions either in early life (such as mild energy restriction in mothers during gestation and lactation) or adulthood (dietary and other challenges), genetic models (mice with ectopic expression of uncoupling protein 1 in muscle, mice with only one functional retinoblastoma protein allele, obesity prone *versus* obesity-resistant mouse strains) and models of pharmacological intervention. **Studies in humans** included: two

randomized placebo-controlled intervention trials with marine n-3 polyunsaturated fatty acids (n-3 PUFA) in obese and non-obese adult subjects, along with postprandial studies at the beginning and end of the intervention studies; a cross-sectional study with adults stratified according to progressive dyslipidaemia; and a study in infants and children stratified according to birth weight and early life diet (formula-fed or breast-fed). A major effort was directed to the characterization of the BIOCLAIMS cohort with 1310 study subjects with full health, a wide range of apparent health, and mildly impaired renal function, which have been characterized for over 500 variables, including vascular status and pro-inflammatory single nucleotide polymorphisms. Selected samples of this cohort were analyzed in the BIOCLAIMS integration (BIG) study aimed at evaluating the most promising candidate biomarkers identified in experimental and human studies in BIOCLAIMS in 4 contrasting groups with slight declines in vascular, metabolic or renal health, as compared with subjects with full health (BIG study and main results from it are summarized in a specific section within this document).

As main results of BIOCLAIMS, the following candidate biomarkers were identified:

- **Transcript-based markers of adverse programming effects and their reversibility by early life leptin supplementation identified through transcriptomic analysis of peripheral blood mononuclear cells (PBMC)**

Within BIOCLAIMS WP2, the offspring of calorie-restricted rats during gestation has been extensively characterized as a model of impaired metabolic health (CRG rats). We have shown that CRG rats: (i) are early programmed for insulin resistance, central leptin resistance and hyperphagia (1); (ii) display reduced expression of sirtuin 1 in key tissues (2); (iii) exhibit a defective sympathetic drive to white adipose tissue (WAT), brown adipose tissue (BAT) and the stomach, leading to reduced thermogenic capacity in BAT and early alterations in plasma ghrelin levels (3-5); and (iv) display alterations in hypothalamic structure and function (6). These changes may contribute to long-term detrimental outcomes of gestational calorie restriction and are partially reversed by forced, stable enhancement of hepatic fatty acid oxidation from a juvenile age (through gene transfer of an active mutant form of carnitine palmitoyltransferase-1a) (7).

Previous studies have linked leptin intake during lactation to the prevention of obesity and the programming of appetite and food preferences later in life and indicated a crucial neurotrophic role of leptin in programming hypothalamic circuit formation (reviewed in (8)). This led us to investigate the capacity of supplementation with physiological doses of leptin throughout lactation to reverse developmental malprogramming in CRG rats. Leptin supplementation to the lactating CRG pups reverted developmental and endocrine defects caused by moderate maternal caloric restriction during gestation (9, 10). Microarray analysis of weaned rat PBMCs identified 224 genes differentially expressed between control and CRG rats, which represent potential early biomarkers of impaired metabolic health, and leptin supplementation reverted most of these changes, normalising 218 out of the 224 genes affected (11). These markers may be useful for early identification and subsequent monitoring of individuals who are at risk of later diseases and would specifically benefit with the

intake of appropriate amounts of leptin during lactation. These results were included in a patent application ("Método para la predicción y/o la prevención de sobrepeso, obesidad y/o sus complicaciones mediante análisis de expresión génica" – "Method for prediction and / or prevention of overweight, obesity and / or its complications through gene expression analysis" (12), and have been extended in an international (PCT) application (13).

- **Transcript-based markers of early priming of metabolic health identified through transcriptomic analysis of PBMC**

Within BIOCLAIMS WP2, the offspring of calorie-restricted rats during lactation has been further characterized as a model of improved metabolic health (CRL rats) (14). CRL rats display lower food intake, body weight and fat accumulation, and improved insulin and leptin signalling throughout life compared to controls. Already at an early age, before other phenotypic changes became evident, CRL rats exhibited differences from control rats in gene expression in tissues such as liver and WAT indicative of an improved ability to handle and store excess dietary fuel. Studies in this model also allowed to propose transcript levels of insulin receptor substrate (IRS1) in WAT and of the leptin receptor (ObRb) and sterol regulatory element-binding protein 1c (SREBP1c) in liver as potential biomarkers of health status in adult rats, on the basis of their correlation with hepatic lipid content, circulating triglycerides and insulin, and insulin resistance indexes (14).

Nevertheless, the emphasis was put on accessible biomarkers by using CRL rats to identify early transcriptome-based biomarkers of metabolic health using PBMC. Microarray analysis of weaned rat (21-day-old) PBMC (performed in collaboration with WP1) identified 278 genes differentially expressed between control and CRL rats, among them a set of genes related to lipid handling including Cpt1a, Fasn, Lipe, Lrp1, Pcyt2, Rxrb, Sorl1 and Star (15). The changes in 6 out of these 8 genes were verified in additional animal models of improved metabolic health at mature age or beneficial metabolic action, namely retinoblastoma protein haploinsufficiency and acute retinoic acid treatment in mice, using total blood cell RNA (16). Additionally, expression levels in total blood of some of these genes (Cpt1a, Pcyt2, and Star) was shown to be responsive to food bioactives of known beneficial effect (resveratrol and hydroxytyrosol) in high fat diet (HFD)-fed mice (17). Collectively, these findings reveal the possibility of using transcript levels of lipid metabolism-related genes in PBMCs or total blood as early biomarkers of metabolic health status.

- **Transcript-based markers of unbalanced diets identified through transcriptomic analysis of PBMC**

Studies in rats developed within WP2 showed that chronic intake of diets with an unbalanced proportion of macronutrients (with excess fat or protein) associates with metabolic and health problems (18). Collaborative work between WP1 and WP2 identified a pool of 7 genes whose expression levels in rat PBMC are affected by the intake of unbalanced diets no matter they are rich in fat or protein, and in the same direction (19). These genes (Cenpn, Dhfr, Acox1, S1pr4, Narg, Copg2 and Eepd1) could be used as a common risk gene signature for unbalanced diets with

disproportioned macronutrient composition. The use of unbalanced diets is increasing in specific groups of population; for that reason, it is of interest to have a gene signature available from an easily obtainable biological material, such as blood cells, to identify deviations from a healthy diet. The impact of unbalanced diets on metabolism has also been characterised through transcriptomic analysis of the liver and other analysis (20, 21). Other studies in WP2 showed that PBMC gene expression can mark early homeostatic imbalance associated with obesity development upon cafeteria diet feeding in rats (22) and homeostatic balance recovery associated with weight loss in obese rats, when reverting from a hyperlipidic to a balanced diet (23).

- **Fasting-refeeding response**

The ability to adapt metabolism from lipid oxidation to carbohydrate oxidation delineates metabolic flexibility and can be used as a measure for metabolic health. This ability can be measured through non-invasive indirect calorimetry (InCa) analysis of the respiratory exchange ratio (RER) during fasting and refeeding. The RER indicates if animals or humans oxidize lipids, which occurs when mammals are fasting (RER 0.7), or carbohydrates, which occurs after a feeding with a diet that contains carbohydrates (RER 1.0). BIOCLAIMS studies within WP1 demonstrate that the fasting-refeeding response can be effectively quantified through InCa analysis and be used as a biomarker for metabolic health sensitive to nutritional interventions, such as the administration of marine long chain omega-3 PUFAs on the top of an obesogenic diet (24) or the consumption of isocaloric diets high in polyunsaturated or saturated fat (both with similar n-3/n-6 ratio) (25). In the latter study, the two diets only induced minor differences in static health markers such as body weight, adiposity, adipose tissue health, serum adipokines or whole body energy balance, nor did we see differences using an (invasive) oral glucose tolerance test. However, the mice fed the isocaloric high saturated fat diet were less flexible in their InCa response to fasting-refeeding, indicating a difference in health status. This was supported by differential lipid accumulation in the liver (25). In another BIOCLAIMS WP1 study, metabolic flexibility at weaning was shown to mark genetically-determined propensity to obesity (26). Increased metabolic flexibility at young age as measured through InCa was also identified in WP2 as an early predictive biomarker of metabolic health in retinoblastoma protein haploinsufficient mice, which were shown to display improved metabolic control as they age and in front of acute metabolic stressors compared with wild-type mice (27). We conclude that measurement of fasting-refeeding response in indirect calorimetry is a sensitive biomarker that can be quantitatively measured and reflects and predicts metabolic health. This biomarker is non-invasive and can be used in humans. In a range of different models, the lack of changes in gene expression in different types of samples (including blood cells) in response to the transition fasting-feeding is emerging as a solid biomarker reflecting the erosion of the metabolic regulation capacity that occurs in the obese state and may be further deteriorate in type 2 diabetes.

- **Acylcarnitines and amino acids plasma metabolome (PM) profile**

Although their significance is not yet well understood, plasma levels of acylcarnitines have been related to insulin resistance and alterations of fatty acid oxidation in tissues.

Acylcarnitine metabolism is intimately connected to amino acid metabolism, especially that of branched-chain amino acids. Animal studies within BIOCLAIMS revealed that the PM targeted on acylcarnitines could be used as a biomarker of (i) metabolic flexibility, i.e. the switch between carbohydrate and lipid metabolism, which is usually assessed using indirect calorimetry during the fasted to re-fed transition (see above); and (ii) propensity to genetically-determined obesity in mice (26, 28). However PM targeted on acylcarnitines was also found to be affected by short- and long-term exposure to dietary fat, acute retinoic acid treatment, and age of the mice in animal studies within the Project. Importantly, fasting levels of acylcarnitines but not amino acids levels marked improvement of both lipid metabolism and inflammatory status in response to marine n-3 PUFA in a human study within BIOCLAIMS (29). Other results indicate that levels of specific amino acids in plasma could serve as early, robust, age-independent biomarker of the propensity to obesity allowing discrimination between low and high body weight gainers over time among mice of identical genetic background (28). We conclude that PM targeted on acylcarnitines and amino acids is a sensitive biomarker with a potential to reveal whole body metabolism and propensity to obesity, however, age and diet represent important variables, which affect the biomarker response.

- **Leptin and Mest**

By using a systematic gene profiling (transcriptomic) approach, a set of genes in WAT which are regulated by HFD feeding and whose short-term expression changes (after 5 days of HFD) are highly predictable for long-term changes (after 12 weeks of HFD) were identified in BIOCLAIMS WP1 (30). We considered this a promising step to establish robust early biomarkers that could shorten animal trials to assess health-promoting food compounds. Out of all short-term predictive changes detected, expression of Leptin and Mest genes in WAT as induced by 5 day of HFD was reversed by three independent nutritional anti-obesity interventions, namely epigallocatechin gallate (EGCG) supplementation, n-3 PUFA supplementation and increased dietary protein (31). Interestingly, Mest mRNA expression changes in WAT were reflected in blood cells, as indicated by studies in collaboration with WP2 (31). Thus, gene expression of Leptin and Mest in WAT and of Mest in blood cells represent suitable biomarkers of adipose tissue expansion sensitive to dietary intervention, which can be useful in the screening and evaluation of bioactive compounds with potential anti-obesity action. In the BIOCLAIMS cohort, leptin increased with age more pronounced in men than women, and a high intake of n-3 PUFAs was associated with lower plasma leptin.

- **Fibroblast growth factor 21 (FGF21)**

Mitochondrial uncoupling in skeletal muscle has become of major interest as a therapeutic target for treatment of obesity, insulin sensitivity, and age-related disease. Within BIOCLAIMS WP1, mice with muscle-specific uncoupling protein 1 (UCP1) transgenic expression have been established as a valuable model for the study of healthy aging (HSA-UCP1 mice, expressing UCP1 under the control of the human skeletal muscle actin promoter). These mice are protected against metabolic syndrome and

reduced life span induced by HFD (32). Closer elucidation of the mechanisms has shown that increased longevity in HSA-UCP1 mice is linked to increased substrate metabolism and induction of the endogenous antioxidant defense system (33). The healthy metabolic phenotype of these mice was also found to be linked to the induction of WAT *browning*, i.e. increased substrate metabolism within typical white fat depots (34). The hormone FGF21 was identified as an endocrine-acting myokine induced by skeletal muscle mitochondrial uncoupling critically involved in the phenotype of HSA-UCP1 mice. Our data indicate that FGF21 secretion by muscle cells is part of a cellular stress response and serves as an endocrine rescue signal for maintaining metabolic homeostasis and health (35). Thus, animal studies in BIOCLAIMS point to FGF21 as a potential biomarker of metabolic robustness. Characterisation of the BIOCLAIMS cohort indicated that FGF21 showed only little day-to-day variability (rated as "excellent reliability") in the absence of metabolic changes.

- **WAT mitochondrial density**

WAT mitochondrial density has been identified as a marker of adipose tissue health on the basis of studies of BIOCLAIMS WP1 in mice revealing increased density following calorie-restricted HFD feeding *versus* HFD feeding *ad libitum* and reduced density following HFD *versus* low fat diet feeding (36). The relations between WAT mitochondria density and various plasma metabolites has been assessed in 4 independent animal studies (3 in WP1 and 1 in WP2) to test if there are circulating metabolites that consistently correlate with WAT mitochondrial density and thus can be used as a metabolite biomarker set for WAT mitochondrial density reflecting WAT health. The main conclusion it that some acylcarnitines (especially C14-2 and C18-2) show consistent significant correlations in the same direction between serum acylcarnitine levels and WAT mitochondrial density in two very different studies (12 week HFD *versus* low fat diet and 12 week HFD *versus* HFD with restricted calories). For C14-2 the changes were in the same range in both studies. Analysis of two short-term studies (6 hour hypoxia (WP1) and 4 day retinoic acid treatment (WP2)) showed changes in serum acylcarnitines, but did not (yet) show changes in mitochondrial density. We conclude that WAT mitochondrial density is a marker that correlates with WAT health. Acylcarnitines reflect metabolic status, but practical limitations prevent their practical use as straightforward surrogate markers for WAT mitochondrial density. The association of C14-2 with WAT mitochondrial density and functioning deserves further attention.

- **Protein damage-related markers**

Damage to macromolecules contributes to cell and tissue dysfunction in ageing and disease. One form of damage is the formation of adducts known as glycation endproducts following dicarbonyl stress, which is the abnormal accumulation of dicarbonyl metabolites such as methylglyoxal leading to increased protein and DNA modification (reviewed in (37)). Methylglyoxal is increased in experimental obesity and appears causally linked to weight gain, inflammation and insulin resistance in obesity. Methylglyoxal is formed during glycolytic and glyceroneogenesis flux, and one

important source of increased methylglyoxal production in obesity is dysfunctional adipose tissue.

Markers of dicarbonyl stress and other forms of damage to the proteome have been measured in samples from various animal and human studies within BIOCLAIMS by WP4, with the aim to investigate the potential of these markers to function as biomarkers of health, healthy ageing, and HFD- or obesity-induced metabolic alterations:

- Studies in BIOCLAIMS have established mice with muscle-specific UCP1 transgenic expression (HSA-UCP1 mice) as a model for healthy aging (see above). Results showed that healthy aging in HSA-UCP1 mice was associated with decreased dicarbonyl stress and oxidative damage in skeletal muscle, likely linked to re-structuring of muscle fibres for resistance to methylglyoxal modification (38). Decreased methylglyoxal modification of these proteins may contribute to increased longevity and healthy ageing.

- We have previously described that moderate maternal calorie restriction during lactation protects offspring against obesity in adulthood (39). Results showed that maternal milk produced during dam moderate caloric restriction had decreased protein glycation, oxidation and amino acids – but not decreased branched chain amino acids (40). Combination of these markers may predict obesity resistance in offspring.

- Within BIOCLAIMS, oxygen restriction (OxR) has been characterized as a non-invasive challenge test that measures the flexibility to adapt metabolism. As part of this characterization, we found that OxR increased serum markers of protein glycation and oxidation in HFD-fed mice, but not in low fat diet-fed mice, a result that contributes to prove the usefulness of the OxR challenge (41)

- The involvement of dicarbonyl stress in HFD-induced decline of pancreatic beta cell function has been investigated. Results indicate that glycation by methylglyoxal likely impairs adhesion of beta-cells to the extracellular matrix in prediabetes *in vivo* and may thereby contribute to beta-cell glucotoxicity and dysfunction with progression to type 2 diabetes (42).

- Results showed that WAT and liver suffer dicarbonyl stress in experimental, HFD-induced obesity, which is exacerbated by decreased activity of the methylglyoxal metabolising enzyme Glo1 in WAT of obese animals. Interestingly, dicarbonyl stress in WAT (but not liver) was prevented in HFD-fed mice with transgenic ectopic expression of UCP1 in WAT, without reversal of decrease in Glo1 activity. Dicarbonyl stress in WAT may be a driver of obesity and in the liver may contribute to hepatic complications - non-alcoholic fatty liver disease (43).

- Protein glycation, oxidation and nitration in clinical obesity and effect of n-3 PUFA supplementation has been investigated in plasma and urine samples of one of the BIOCLAIMS human intervention studies (44). The results revealed no increase in plasma protein damage markers but increase in dysglycaemia markers and oxidative and dicarbonyl stress-related damage in urine of obese subjects. Administration of n-3 PUFA for three months decreased markers of dysglycaemia and oxidative damage in urine. Urinary dysglycaemia and dicarbonyl stress markers are high abundance, easily

measured, non-invasive biomarkers of two independent risk factors of decline in metabolic health (insulin resistance and dysfunction of adipose tissue) that may find future clinical utility in health assessments in obesity (44).

- **Lysophospholipids and taurocholic acid as plasma markers of dyslipidemia and atherogenic plaque formation**

A model of progressive dyslipidemia based on HFD feeding for different lengths of time was developed within BIOCLAIMS WP2 in hamsters. A chemical-induced model of dyslipidemia (intraperitoneal treatment of hamsters with poloxamer 407) was also studied, to allow distinguishing biomarkers of dyslipidemia and of HFD feeding *per se.* Results of plasma non-targeted metabolomics (Liquid chromatography - tandem mass spectrometry) indicated that plasma lysophospholipids are sensitive to alterations of the lipid metabolism that accompany the progression of dyslipidemia and, therefore, could be proposed as biomarkers of this pathology in hamsters. In particular, higher circulating levels of lysophosphatidylethanolamines 20:4 and 20:6 were found in both HFD-fed and poloxamer 407-treated hamsters at 4 days. Therefore, these metabolites could be suggested as early biomarkers of dyslipidemia. Levels of lysophosphatidylcholine 20:2 were also of interest as early markers of dyslipidemia as identified in two independent experiments using the dietary model. Lipidomic analysis in plasma, liver and aorta samples following HFD and poloxamer 407 treatments has been performed. The lipidomic analysis of plasma revealed other biomarkers of dyslipidemia at 30 days, mainly belonging to the categories of diacylglycerols, phosphatidylethanolamines, phosphatidylserines and triglycerides. Detailed analysis of the results obtained at 4 days is ongoing to establish if some of these biomarkers could indicate the onset of dyslipidemia.

To test the translation of the above finding to humans, lysophospholipids were analysed in human samples from two independent human studies within BIOCLAIMS, one focused on dyslipidemia and one focused on obesity. In the first study, volunteers − all with blood triglycerides lower than 150 mg/dL and not taken hypolipidemic drugs − were stratified in three groups according to their LDL-cholesterol (LDL-c) levels: optimal profile group (LDL-c <100 mg/dL), near optimal profile group (LDL-c 100-129 mg/dL) and borderline-high profile group (LDL-c 130-189 mg/dL). The results revealed that levels of lysophospholipids in plasma change as dyslipidemia progresses, and that the changes are group-dependent: lysophosphatidylethanolamines changed in the near optimal group, and lysophosphatidylcholines and lysophosphatidylinositols changed in the borderline- high group. Results of the human study focused on obesity revealed that obesity induces clear changes in the plasma lysophospholipids profile, and that an acute lipid challenge magnifies the differences between normoweight and obese subjects (45). Thus plasma lysophospholipids are sensitive to two common alterations of lipid metabolism, namely dyslipidemia and obesity. We conclude that plasma lysophospholipids monitoring might be useful for detecting early alterations in the metabolism of lipids leading to obesity or dyslipidemia. However, more research is still needed in order to establish the origin and cause of the observed changes.

Other studies in hamsters revealed that the plasma metabolome is sensitive to the intake of grape seed procyanidins polyphenols in the context of dyslipidemia (46). An additional study in hamsters allowed identifying taurocholic acid as a potential plasma biomarker of early atheromatous plaque formation responsive to grape seed extract consumption (47). In the later study, a more potent high fat high cholesterol challenge was used, since the objective was to induce atherogenesis. The levels of taurocholic acid in plasma directly correlated with the levels of free cholesterol in the aorta, a known biomarker of early atherogenesis, and the administration of the polyphenol-rich extract counteracted the increase in taurocholic acid in the HFD-fed animals.

- **Other potential biomarkers of interest identified in animal studies**

WAT expandability and adiponectinemia emerged as potential markers of a good metabolic profile explaining the resistance of female mice (as compared to males) to the adverse metabolic consequences of a HFD (48). Results obtained in a model of nutritional intervention in early life indicated, in turn, that WAT expandability is sensitive to early life micronutrients intake (in particular vitamin A) that increase the proliferative status of adipocytes at weaning (49). Other results in models of nutritional intervention in early life suggested plasma fatty acid profile as an early complex marker of sex-dependent propensity to obesity, associated to maternal leucine supplementation during lactation in rats (50, 51).

- **TLR4 expressing monocytes as a novel potential marker of inflammation associated to human obesity**

Although many blood markers of inflammation have been proposed, clear recommendations of which biomarkers to use and how to interpret patterns of biomarkers and changes in biomarkers in the context of nutritional studies in relation to inflammation are lacking (52). There is wide variation in any of the soluble or blood cellular markers of inflammation even amongst normal, fairly healthy individuals. Moreover, since the inflammatory response is dynamic, it is likely that a combination of multiple inflammatory markers and integrated readouts based on kinetic analysis following challenge will be the most informative biomarker of inflammation. Toll-like receptors (TLRs) are membrane receptors involved in the downstream modulation of inflammatory pathways in blood cells and other cells and in innate immunity. A human study within BIOCLAIMS WP3 designed to identify whether n-3 PUFA are anti-inflammatory in both normal weight and obese subjects revealed a greater number of TLR4 expressing monocytes (and fewer TLR2 expressing monocytes) in blood of obese subjects than normal weight subjects, together with higher blood concentrations of inflammatory markers and leptin and lower of adiponectin in the obese. Moreover, obese subjects showed exaggerated metabolic responses (greater increases in blood triglycerides, glucose, non-esterified free fatty acids and insulin) and a higher level of inflammation following consumption of a high fat meal used as a challenge, and they also showed an elevation in TLR4 expressing monocytes in the hours after the high fat meal challenge that was not seen in normal weight subjects. These are novel findings that point to TLR4 as a novel potential marker of inflammation associated to human obesity (53). In this study, acute beneficial effects of n-3 PUFA on the post-prandial

response to the high fat meal and positive effects of chronic (12 weeks) n-3 PUFA intake on metabolic and inflammatory parameters were only seen in the normal weight group.

- **Glucose-dependent insulinotropic polypeptide (GIP) as a potential new biomarker of metabolic risk in obesity sensitive to nutritional intervention**

GIP is a hormone secreted from the gut mucosa that stimulates glucose-dependent insulin secretion from pancreatic beta-cells. Besides this insulinotropic effect, experimental studies have implicated GIP as a modulator of lipid metabolism favouring fat deposition in adipose tissue, muscles and liver. Altered GIP secretion and action was recently linked to obesity-related metabolic disorders but there are conflicting results and underlying mechanisms are not well understood. GIP has been investigated in a human study in BIOCLAIMS examining the combined effect of 3 month mild low-calorie diet and marine n-3 PUFA supplementation (1.8 g/day n-3 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in a ratio of 5:1) in obese, non-diabetic subjects. This study also included groups of non-obese subjects under isocaloric diet and receiving either placebo or the n-3 PUFA capsules. Postprandial responses to acute fat and glucose challenges at baseline and after the 3-month intervention were monitored. Fasting circulating GIP levels were found to be higher in the obese subjects than the non-obese subjects, and to correlate directly with a series of metabolic and inflammatory risk markers (glucose, triglycerides, total- and LDL-cholesterol, as well as sE-selectin, MCP-1, visfatin and leptin/adiponectin ratio) and with insulin resistance index (HOMA-IR) in obese subjects. Moreover, an increased release of GIP after the fat or the glucose challenge correlated with an increased postprandial response of glucose, triglycerides and free fatty acids, and with reduced postprandial antioxidant (glutathione peroxidase) activity. Caloric restriction in combination with n-3 PUFA (but not placebo) supplementation resulted in reduced fasting GIP and reduced GIP release in response to glucose, and the combination had beneficial metabolic effects over caloric restriction alone in the obese subjects on parameters including fasting insulin, insulin resistance index, insulin output during glucose challenge and fasting triglycerides. Supplementation of low calorie diet with n-3 PUFA significantly improved plasma lipid profile (in terms of reduction of n-6 PUFA) in obese subjects characterized by high GIP plasma levels. From these results, GIP is proposed as an early biomarker of metabolic and inflammatory complications of obesity and a predictive factor of beneficial effects of n-3 PUFA supplementation (54, 55).

- **Carboxylated and undercaborxylated osteocalcin**

Carboxylated osteocalcin interacts with Ca^{2+} and participates in bone remodelling, whereas undercarboxylated osteocalcin has a hormone-like function in energy metabolism and other processes. Disruption of osteocalcin signalling leads to glucose intolerance in mice, which was also recently demonstrated in humans. Within BIOCLAIMS WP3, levels of carboxylated and undercarboxylated osteocalcin have been measured in non-obese subjects, healthy obese subjects and obese prediabetic subjects before and after n-3 PUFA supplementation (as above). Obese subjects presented lower levels of carboxylated osteocalcin compared with non-obese subjects.

Correlation analyses indicated lower levels of carboxylated osteocalcin to be associated with increased levels of inflammatory markers in blood (hsCRP and visfatin). Obese subjects with higher than median value of carboxylated osteocalcin had lower levels of inflammatory markers. Obese prediabetic subjects presented lower levels of undercarboxylated osteocalcin compared with healthy obese volunteers. Correlation analyses indicated lower levels of undercarboxylated osteocalcin to be associated with increased fasting insulin and insulin resistance index (HOMA-IR). Obese subjects with higher than median value of undercarboxylated osteocalcin had a better insulin resistance profile (lower fasting insulin levels and HOMA-IR). n-3 PUFA intake for 3 months resulted in increased carboxylated osteocalcin. From these results, we suggest that decreased blood concentration of carboxylated osteocalcin (Gla-OC) marks low grade inflammation accompanying human obesity, whereas decreased undercarboxylated osteocalcin (Glu-OC) marks insulin resistance development in obesity (56).

- **n-3 PUFA-derived lipid mediators resolvins as biomarkers of anti-inflammatory response to long-chain n-3 PUFA supplementation**

Obese women responded to n-3 PUFA (DHA/EPA) supplementation with a decrease of circulating levels of pro-inflammatory markers (hsCRP, sEselectin, sVCAM, sPECAM, MCP1, resistin) and an increase of circulating levels anti-inflammatory n-3 PUFA (mainly DHA)-derived resolvins (RvD1, RvD2 and PDX). Microarray analysis of blood polymorphonuclear cells before and after the dietary intervention showed that n-3 PUFA supplementation did not change gene expression of enzymes related to resolvins synthesis but up-regulated the expression of resolvin RvD1 receptor. Subsequent pathway analysis of the microarray data indicated n-3 PUFA activation of peroxisome proliferator activated receptor alpha (PPARα) and nuclear factor erythroid 2-related factor 2 (Nrf2) target genes related to beta-oxidation of fatty acids, phospholipid synthesis (plasmalogens, cardiolipins, phosphatidylinositols), mitochondria electron transport chain proteins, and antioxidant enzymes, together with decreased expression of nuclear factor kappa B (NF-κB) target genes related to inflammation (inflammatory cytokines). From these results, n-3 PUFA-derived resolvins are proposed as biomarkers of anti-inflammatory effect after DHA/EPA supplementation (57).

- **Studies on NF-κB activation**

NF-κB, a redox-sensitive transcription factor was selected for study in BIOCLAIMS as an interface between inflammation and oxidative stress. Activated NF-κB translocates to the nucleus and binds to the κB binding site in the regulatory region of different genes encoding for central regulators of inflammatory processes. Highly specific NF-κB binding activity in whole cell extracts of PBMC was determined for the p65-p50 heterodimer in the entire BIOCLAIMS cohort. Associations with subject characteristics and other biomarkers were jeopardized by frequently used drugs (such as antihypertensives, acetylicsalicylic acid, statins, oral antidiabetics), with an incremental decrease in p65 activation with the number of NF-κB inhibiting drugs, while p50 activation did not differ. Age and gender did not have an effect on NF-κB.

BIOCLAIMS integration (BIG) study

BIG study aimed at evaluating the most promising candidate biomarkers identified in experimental and human studies in BIOCLAIMS in human subjects with early declines in different aspects of health as compared with healthy subjects, all of them selected from the BIOCLAIMS cohort built as part of Project WP3 work. Four contrasting groups were selected from this cohort: participants with 1) mildly impaired renal function, based on estimated glomerular filtration rate; 2) impaired vascular health, as evidenced by intima-media thickness higher than 75% of the healthy population; 3) impaired metabolic health, as evidenced by elevated HOMA index and elevated glycated hemoglobin A1c; 4) Healthy subjects. In these groups, most promising biomarkers for validation derived from animal studies as well as human studies in BIOCLAIMS were measured, generally by partners firstly involved in the biomarker identification. The biomarkers selected were: protein damage markers in urine; plasma metabolome profile (acylcarnitines and aminoacids); lysophospholipids in plasma; taurocholic acid in plasma; FGF21 in serum; gene expression of selected lipid metabolism-related genes in PBMCs; red blood cells and plasma fatty acid profile; carboxylated and undercarboxylated osteocalcin in serum; plasma leptin/adiponectin ratio; activation status of NF-kB in PBMC; and plasma oxidative markers (mercaptalbumin and non-mercaptalbumin). In addition, microarrays for the analysis of while genome transcriptome of PBMCs have been carried out for the vascular and metabolic health compromised groups as compared to healthy subjects. Overall, these analyses have engaged all BIOCLAIMS partners.

In this study, the contrasting groups with impaired health were differentiated from healthy subjects by several biomarkers. Different components of the urinary modified amino acid metabolome (glycated, oxidised, nitrated and crosslinked amino acids), normalised to creatinine, were characteristically changed in early-stage impaired metabolic health, impaired vascular health and impaired renal health. FGF21 was elevated in subjects with impaired renal and metabolic health compared to healthy subjects. 21/31 acylcarnitines differed significantly between subjects with mildly impaired renal health and the 3 other groups. Expression of specific genes in blood cells was elevated in the groups with impaired health compared to full health; a gene in particular marked specifically the impaired renal health group and its expression in blood cells correlated with known markers of this condition. Circulating lysophospholipids were altered specifically in the group with impaired renal health. Overall, the group with mildly impaired renal health was recognized as different from the other groups by most of the biomarkers, but the other groups differed also significantly from healthy subjects. Given that impaired renal function is a silent disease, early detection of these metabolic consequences will have major health implication. An index to be used as a screening tool is currently under evaluation.

Contribution of BIOCLAIMS to the characterization or set up of novel samples, challenges, network tools and methods of interest for research on biomarkers in nutrition and health

- BIOCLAIMS has placed special emphasis on developing biomarkers using samples that are minimally invasive, with a low subject burden. In particular, BIOCLAIMS has contributed basic knowledge that substantiates the **use of blood cells** as a source of transcript-based biomarkers for nutrition and health related aspects. Findings in BIOCLAIMS support blood cell gene expression as a source of biomarkers of early priming of metabolic health (11, 15), homeostatic alterations caused by the intake of unbalanced diets (19), and homeostatic balance recovery associated with weight loss in obese rats (23). Expression levels of some of the blood transcript markers identified in animal models were found altered in human subjects with impaired health. Studies within BIOCLAIMS also support the use of blood cells as a source of transcript-based markers of beneficial responses to interventions with specific nutrients or food supplements. Gene expression in PBMC captured reversibility by early life leptin supplementation of adverse programming effects of maternal calorie restriction during gestation in rats (11). It was also shown to capture alterations in lipid and cholesterol metabolisms induced by a HFD in hamsters and their amelioration by grape seed procyanidins oral supplementation (58). Gene expression in total blood of mice was shown to reflect known transcriptional responses to retinoic acid treatment in liver and adipose cells (16). Importantly, we also show that blood cell transcriptomics in humans can mark anti-inflammatory n-3 PUFA action (57) and be used as a source of potential biomarkers of articular health improvement following glycosaminoglycan intake (59). BIOCLAIMS has also contributed novel knowledge substantiating **plasma metabolome** and **damage to the proteome** in accessible body fluids as biomarkers of health or early decline of health.

- Challenge tests to stress homeostasis may reveal deviations in health that remain masked under unchallenged conditions. Besides applying a variety of classical challenge tests, BIOCLAIMS has developed and proven the relevance and sensitivity of **non-invasive, indirect calorimetry (InCa)-based challenge tests**, in particular InCa analysis of the response to oxygen restriction and to fasting-refeeding, which measure the flexibility to adapt metabolism to these varying conditions. These tests have proven to be more useful than static health markers and classical invasive challenge tests, such as the oral glucose tolerance test, to reveal differences in health status between young and aged, but otherwise healthy, mice (60); mice after short-term (5 days) high fat versus low fat diet feeding (41); mice fed isocaloric diets differing in lipid compositions (unsaturated *versus* saturated, but with similar n-6/n-3 ratio) (25); and mice differing in their genetic predisposition to obesity (obesity-prone B6 *versus* obesity- resistant A/J strains) (26). The unique **oxygen restriction challenge test** developed within BIOCLAIMS (based on ambient, mild normobaric hypoxia, i.e. 12-14% instead of 21% O_2), also revealed diet- and age-dependent differences in molecular responses in liver and adipose tissues, as well as in various serum parameters (41, 60), and has been successfully adapted for application to humans.

- The **BIOCLAIMS cohort**, 606 men and 704 women, aged 18-85 years, has been extensively characterized using established dietary, clinical, anthropometric, and biochemical biomarkers along with the plasma metabolome, adipokines, FGF-21, activation of NF-kB in PBMC and vascular status. Effects of age, gender, menopause as well as menstrual cycle, season and day-to-day variability have been mapped for these biomarkers. This cohort, for which a sample collection is still available, will be useful for future biomarker research.

- A **BIOCLAIMS nutritional phenotype database** has been implemented (WP5) and populated with BIOCLAIMS studies (WP1-WP3). A reference network of adipose tissue health was generated by WP5 from data including BIOCLAIMS studies in mice, rats and humans. This **White Adipose Tissue Health Reference Network (WATRefNet)** was constructed as a resource for discovery and prioritization of mechanism-based biomarkers for WAT health status and the effect of food and drug compounds on WAT health status (61). The WATRefNet represents a sustainable knowledge resource for extraction of relevant relationships such as mechanisms of action, nutrient intervention targets and biomarkers and for assessment of health effects for support of health claims made on food products.

- Key variables in experimental studies on obesity and metabolic health in rodents that can influence many processes and outcomes have been addressed. **A BIOCLAIMS reference semi-purified rodent diet** was formulated early during the development of the Project to facilitate direct comparison among animal studies within the consortium and to function as a reference diet for nutritional physiology (62). The impact of ambient temperature on HFD-induced obesity and metabolic damage in B6 mice was also studied (63).

- A fast and sensitive Ultra performance liquid chromatography - tandem mass spectrometry **method for the analysis of acylcarnitine and amino acid profiles** from minute amounts of plasma has been implemented and used in the frame of different BIOCLAIMS studies. **Advanced methods for the assay of oxidized and glycated proteins** and nucleotides and the measurement of methylglyoxal in physiological samples have been published in top journals, thus contributing to extend the use of these markers in clinical and nutrition research (64-66).

Contributions of BIOCLAIMS to mechanisms of dietary factors and basic science

In seeking for biomarkers of health effects of dietary factors, BIOCLAIMS has contributed novel basic knowledge regarding mechanisms and biological targets. The following are illustrative examples:

- The search of biomarkers of endothelial cell vitality reflecting the vascular health beneficial impact of dietary bioactives from fruits and vegetables led to the proposal within BIOCLAIMS of a novel model of regulation of the transcription factor Nrf2 (67, 68). Nrf2 controls the cellular expression of a battery of protective genes countering oxidative stress, toxic substances, lipid peroxidation, inflammation, metabolic dysfunction and ageing. The Nrf2 system senses challenge to homeostasis in the cell

cytoplasm and activates a protective transcriptional response. Such stress-responsive signalling is vital in resisting oxidative damage, dicarbonyl glycation damage, cell dysfunction, cytotoxicity and mutagenesis, thereby contributing to decreasing risk of metabolic and vascular disease. A key feature of current hypothesis for activation of the Nrf2 system is accumulation of Nrf2 protein in stimulated cells. Our initial studies suggested that whilst this occurs at supra-physiological and toxic concentrations of Nrf2 activators, it does not occur with physiological and non-toxic activator exposures. Molecular characterisation of the mechanism of Nrf2 regulation in vascular endothelial cells *in vitro* led BIOCLAIMS scientists to propose a revised model in which Nrf2 undergoes translocational oscillations between cytoplasm and nucleus regulated by post-translational modifications of Nrf2. Increased oscillation frequency facilitates both increased transcriptional response and increased surveillance of the cell stress state when homeostasis is challenged. Improved understanding of regulation of the Nrf2 systems will lead to improved selection of health protection linked biomarkers and improved manipulation of the Nrf2 system would likely lead to more effective deployment of micronutrient Nrf2 activators in functional foods and improved prevention of diabetes and cardiovascular disease. Bioactive screening may be improved by addressing this mechanism. Control of Nrf2 activation at multiple steps suggests synergistic combinations of bioactives are likely most effective in achieving health benefit.

- Cellular mechanism linking mitochondrial uncoupling in skeletal muscle to the metabolic changes which ultimately affect whole body metabolism and lead to the healthy aging phenotype that characterizes HSA-UCP1 mice (which express UCP1 ectopically in skeletal muscle cells) have been analysed in depth (35, 69). This analysis revealed a remodelling of skeletal muscle due to muscle mitohormesis promoting cellular survival via serine/glycine pathway flux, and largely independent of AMP-activated protein kinase (AMPK) activation.

- Studies within BIOCLAIMS have contributed basic knowledge regarding biological targets and mechanisms connecting early life nutrition with the programming of obesity and metabolic health in adulthood. Targets and mechanisms of this nutritional programming revealed in BIOCLAIMS studies (mainly in the offspring of dams submitted to caloric restriction during gestation or lactation) include the development of hypothalamic structures (9), sympathetic innervation to adipose tissues (both WAT and BAT) and the stomach (3-5), sirtuin 1 signalling in peripheral tissues (2), central and peripheral leptin and insulin sensitivity (1, 14), and adipose tissue cellularity (49). Besides calorie restriction during gestation or lactation, additional nutritional conditions in the perinatal period have been assayed in rats within the Project including: maternal HFD consumption (70), maternal perinatal supplementation with different fat sources (71, 72), leucine supplementation to lactating dams (50, 51), moderate vitamin A supplementation to pups (49), and post-weaning cafeteria diet in young rats (73). While the focus of most of these studies has been metabolic programming, impact on other end-points such as exercise performance (70), food behaviour including preferences (8) and other behavioural changes(73) has been also addressed.

- Within the scope of BIOCLAIMS, novel targets of positive metabolic effects of a variety of nutrients including n-3 phospholipids from fish (74), n-3 PUFA (75, 76), oleic acid versus its trans isomer elaidic acid (77), other fatty acids (78), conjugated linoleic acid (79, 80), dietary polyphenols (75, 81, 82), and leptin (83) have been explored.

- Review articles have been produced related to nutritional perinatal programming of body weight control and obesity (84-86); mechanisms and nutritional control of mitochondrial uncoupling and *browning* of WAT (87-89), which are important processes related to energy balance and metabolic health; dicarbonyl stress in cell and tissue dysfunction contributing to ageing and disease (37); and challenges in obesity research (90).

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Part 4. The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

The overarching theme of BIOCLAIMS, to translate the data generated from large-scale efforts in biomarker research into information of relevance for supporting future health claims of foods or supporting new or existing EU directives or policy decisions, has **important societal and economic implications**. Stakeholders interested in biomarkers and results of the BIOCLAIMS project include agro-food industry and policy makers, general public and research community.

To promote the societal and economic impact of BIOCLAIMS, integrative efforts have been conducted for the dissemination, exploitation and transfer of the project results to stakeholders through specific work packages within the Consortium (WP5, WP6, WP7) in tight connection with WP8, responsible of project coordination and management, according to the initial plan for the use and dissemination of foreground included in the project application and DoW. In keeping with the Communication Plan established at an early stage of the Project, all partners together with the Project Coordinator have been engaged in the dissemination of the Project concept and results in different scientific and socio-economical forums, through lectures, interviews in TV/radio, webcasts and news in newspapers and popularizing journals.

The important impact achieved by BIOCLAIMS in the different areas concerned is demonstrated by:

- the fluent interaction maintained with the agro-food industry, as well as European organizations and regulatory agencies dealing with food and health issues;
- the presence of BIOCLAIMS in public media and other efforts directed to increase general public awareness on issues and novel developments in the food and health arena, with more than 3000 entries for "Bioclaims" in Google.
- the many relevant scientific collaborations, exchanges, publications as well as presentations and communications to top scientific meetings by BIOCLAIMS partners produced as part of the Project development;
- the training activities carried out to form young scientists in nutritional and biomarker research, which have resulted in the presentation so far of 11 doctoral thesis developed within the Project;
- the generation of a collection of exploitable foregrounds, including a patent application on a nutrigenomics-derived biomarker.

These different points are further addressed in the next headings on BIOCLAIMS dissemination activities, training activities and exploitation of results.

Dissemination activities

a) Scientific symposia and meetings

BIOCLAIMS has organized two main Symposia to promote the establishment of contacts with stakeholders interested in nutrition-related biomarkers and results of our project:

- the **"First BIOCLAIMS symposium on biomarkers of robustness in nutrition research",** hold in Tarragona (Spain) on 8-9 November 2011. This symposium was intended for discussion and dissemination of concepts and practices of biomarkers for robustness within nutrition research practice. It included presentations from BIOCLAIMS members, international scientists and food industry, and a discussion session about challenges, opportunities, research needs, and implementation strategies in nutrition research. This meeting counted with the active participation and contributions of a selected group of representatives from main companies and European organizations such as Nestle, DSM and ILSI, which were following the Project from the beginning with interest.

- the symposium "**Biomarkers and health claims on Food - BIOCLAIMS meeting with stakeholders"**, hold in Palma de Mallorca, Spain from 12 to 13 February 2015, when the Project was about to formally end. This symposium was intended to present and discuss the new generation of biomarkers developed within BIOCLAIMS with stakeholders of the scientific community, food industry and regulatory agencies aiming at optimal acceptance of the developed biomarkers. It was addressed to policy regulators, representatives from leading food and health industry as well as SMEs, and marketing and law specialists on food aiming at gather increasing knowledge of the developed biomarkers and the regulations that affect biomarkers and health claims. It counted with the participation of members of the EFSA NDA Panel competent on Health claims, including the vice-chair of the Panel and the Chair of WG-Claims of EFSA; industrial representatives from relevant European Food Companies including Unilever, Danone-Nutricia and Lactalis-Puleva, among others; the head of the food law gabinet of the Federation of Food and Drink industries in Spain, and directors of outstanding European gabinets of attorneys specialized in food issues, together with BIOCLAIMS scientists.

In addition to the two aforementioned symposia, BIOCLAIMS initiated or was involved in the organization of six additional symposia, usually joint to BIOCLAIMS internal meetings. These included our participation as organizers and speakers in the **Phenotypic Flexibility Symposium** (El Escorial, Spain, February 4-6, 2013), a symposium focused on flexibility as characteristic of optimal health, its mechanisms, processes, role of diet and nutrition, consequences for healthy ageing and disease, its use as biomarker and its importance for food industry. They also included our participation in the **IUNS 20th International Congress of Nutrition** (Granada, Spain, September 7-15, 2013), one of the most important congresses in Nutrition, in which BIOCLAIMS results were presented in seven invited talks within the session "New biomarkers for health claims made on food" which was organized by BIOCLAIMS, plus one invited talk to the session "OMICS technologies with nutritional perspectives".

Communication with stakeholders also comes from contracts external to the Project and established contacts of partners with companies that are interested in the BIOCLAIMS results as JUMC with FMC Corporation; WU with Nutricia Research and Friesland Campina; UWA with Unilever; CTNS with Laboratorios Ordesa and Andres Pintaluba; UIB with Unilever, CocaCola and Bioibérica; USoton with EPAX, Pronova and Danone. One of the partners (UIB) has promoted and participates in a spin-off technology based company being interested in BIOCLAIMS results (Alimentomica).

b) Publications and participation in scientific meetings

So far (until April 2015), results obtained within BIOCLAIMS led to 63 already published or accepted articles most of them (51) in highly relevant, top-ten (18) or first quartile (33) journals in their category, and 11 submitted manuscripts. Importantly, 21 of these published or submitted papers are joint publications of two or more BIOCLAIMS partners. There are many additional publications derived from BIOCLAIMS which are currently in preparation, that are expected to be submitted within the next weeks and months. Many of these are joint publications, resulting from the assessment of promising candidate biomarkers developed by individual partners in additional, mostly human, samples from other studies within the Consortium, which has just finished.

Leading scientists of BIOCLAIMS have given a vast number of invited lectures about their projects and results as well as methodologies related to BIOCLAIMS in national and international top scientific meetings. This include the dissemination among the scientific community of the concept of Health Benefit Reference Networks, as a knowledge resource for finding novel and prioritizing known biomarkers of health status, discovery of their mechanistic links to the health benefit endpoint and for assessment of health effects of nutrient interventions. Also, there were many posters coming from the different partners presented in international meetings and congresses.

c) Presence in public media

The Coordinator of BIOCLAIMS, Prof. Dr. Andreu Palou, and other leading scientists of BIOCLAIMS have reported in press, radio and TV interviews and broadcasts about their work related to BIOCLAIMS and about BIOCLAIMS concept, goals and achievements. Many press releases and features referring to BIOCLAIMS research issues have appeared in printed and electronic national and local newspapers and magazines of general scope or focused on health, food, and business, mainly in Spain and UK. Information has been disseminated through webcasts and newspaper articles to a lay public on a national level. There has also been direct interaction of partners with the general public in special events such as scientific nights which has contributed to disseminate BIOCLAIMS.

Scientists of BIOCLAIMS have been awarded important international or national scientific awards for their studies including studies that have received support from BIOCLAIMS. The Coordinator of BIOCLAIMS, Andreu Palou was recognized in 2013 with the XXIII DuPont Science Award for his scientific contributions in the field of molecular nutrition: the regulation system of body weight (obesity) Leptin and the

relationship between diet and (epi) genetic (Nutrigenomics and personalized nutrition), the diet / disease mechanisms related to food Safety and Efficacy, Functional Food, and the identification of health claims on foods and new biomarkers for European substantiation of health claims in food. In 2013, Jan Kopecky (the group leader at ASCR) obtained an award by the Minister of Education, Youth and Sports of the Czech Republic for his studies of "New mechanisms in the complex effects of omega-3 fatty acids: perspectives for health", based on 12 articles published recently. Several of these publications were supported by BIOCLAIMS. These awards were commented in local newspapers, as well on several websites in Spain, Czech Republic and international.

Training activities

Training of young scientists has been a major activity and goal within BIOCLAIMS. Undergraduate as well as graduate students and young postdoctoral scientists were involved in the experiments and analyses performed within the different BIOCLAIMS projects. Over 150 students and young scientists (80% of them female) have received training within BIOCLAIMS.

Eleven Doctoral Theses have been completed so far developed under the framework of BIOCLAIMS at partners DIfE, WU, UIB, USoton and ASCR.

BIOCLAIMS was involved in the organization of three Annual Doctoral Workshops on Molecular Nutrition from 2012 to 2014 as a regular training and exchange platform. These workshops were organized at the University of Tarragona, Spain. These workshops aimed at PhD students and researchers within the field of Molecular Nutrition and Physiology to discuss on recent findings, technologies, etc. and to offer the PhD candidates educational courses as well as offer an environment to stimulate cooperation in terms of research and education. They thus provided excellent opportunities for interactions between doctoral students and senior and experienced European scientists in the field.

An additional training opportunity was the Erasmus Intensive Programme TOOLTIPS (Tools Targeted on Obesity Intervention and Prevention Strategies for Efficient and Sustained Implementation), which was organized by University of Graz from January 22-February 5, 2012 at Grundlsee, Austria. This was a two-week intensive course with teachers and students from across Europe who gathered to teach and learn how to develop tools for obesity research including, among other topics, biobanking, tool assessment, biomarker research and risk assessment, issues relevant to BIOCLAIMS. Five BIOCLAIMS members gave lectures at this workshop.

Exchange visits for training as well as for transfer of expertise within the consortium have been helped and encouraged. Overall, there were 29 exchanges between BIOCLAIMS partners and 26 exchanges between BIOCLAIMS partners and external partners dedicated to research or training. These exchanges ranged from short term visits of a few days up to internships of undergraduate and PhD students for up to 3 months.

Exploitation of results

BIOCLAIMS has considered from the very beginning the issue of exploitation of project results.

Next follows the most promising results identified by the partners:

- The identification of a group of genes, whose expression levels in blood cells may be indicative of programmed susceptibility to obesity-related chronic diseases, and whose alterations can be reverted by leptin supplementation during the suckling period. These markers, once confirmed in humans, may be useful for early identification and subsequent monitoring of individuals who are at risk of later diseases and would specifically benefit from the intake of appropriate amounts of leptin during lactation. These results have been included in a patent application of the UIB partner entitled "Method for the prediction and or prevention of overweight, obesity and/or its complications by gene expression analyses". (P201430428, Spain) (Priority data: 26 March 2014). The PCT application has been presented. These results are susceptible of being licensed and have the potential to be used to substantiate the beneficial effects of nutrients or bioactive compounds for infants. The UIB has created a spin-off called Alimentomica that could exploit the content of the patent.
- New effects of n-3 PUFA on incretin response to a meal that could lead to "improved satiety" claim.
- WAT mitochondrial density can be used as a marker for WAT health status and may be used towards substantiation of health claims made on food.
- The validation of the use of Mest/Peg1 gene expression in white fat or whole blood as a marker of adipose tissue expansion for use in screening studies for dietary anti-obesity compounds.
- The confirmation that lysophospholipids are sensitive to two common alterations of the lipid metabolism such as dyslipidaemia and obesity (based on animal and human studies). Therefore, the analysis of these metabolites might be useful for detecting early alterations in the metabolism of lipids that could lead to these pathologies. Lysophospholipids could be used as a biomarker for dyslipidaemia and may be used towards substantiation of health claims made on food.
- Results from a large cohort of mice markedly differing in their body weight gain induced by high-fat feeding revealed that plasma metabolome focused on acylcarnitines and amino acids could be used as a gender-specific complex biomarker of propensity to obesity and possibly also as a biomarker of obesity-associated metabolic disorders.
- A combination of protein damage biomarkers discovered in urine is able to detect and discriminate between good health and early decline in metabolic, vascular and renal health in humans. This paves the way for a non-invasive laboratory-based test for screening and assessment of early-stage decline in

glucose tolerance, decline in kidney function and increased risk of cardiovascular disease. The BIOCLAIMS consortium is considering IPR protection.

- Role of Nrf2 as a dynamic stress sensor and regulator of health protective gene expression. Studies of regulation of the transcriptional factor Nrf2 contributing to the health beneficial effects of fruits and vegetables, including mathematical systems modelling, revealed this novel function of Nrf2. Screening of health beneficial dietary bioactive substances was thereby improved and a rational basis for synergistic combination of dietary bioactives emerged.
- A number of results generated by the consortium are under review and they could also be confirmed and validated in a near future. Between those, the preliminary results about the early biomarkers carboxylated and undercarboxylated (Gla-/Glu-) osteocalcin of prediabetes aggravation are going to be assessed in a last data analysis; new markers of resistance to low-grade inflammation (resolvins, GIP, osteocalcin) modified by PUFA are under examination; among other new advances.

Additionally, the consortium has produced new analytical methodologies and applications to evaluate biomarkers efficacy or to detect these biomarkers as:

- Analysis of the response to oxygen restriction by indirect calorimetry is a promising new method to test food products on potential beneficial effects for metabolic health.
- Indirect calorimetry based challenge tests (during fasting and re-feeding or oxygen restriction) allow quantification of health status and are more sensitive than many classic serum biomarkers to reveal differences in health status.
- A diagnostic kit involving a core set of early biomarkers contributing to prevent obesity could be designed.
- Urinary amino acid metabolome analysis by stable isotopic dilution analysis using liquid chromatography with tandem mass spectrometry for screening health impairment.
- A novel method for the analysis of acylcarnitines and amino acid profiles in plasma has been developed, which is faster and more sensitive than a previously used method based on the Biocrates kit.
- Advanced methods for the assay of oxidized and glycated proteins and nucleotides and the measurement of methylglyoxal in physiological samples.

The “BIOCLAIMS meeting with stakeholders” of February 2015 in Palma de Mallorca (Spain) allowed the Consortium to share its main results but also the associated concerns about IP (protection of biomarkers for health claims) with representatives from the European Commission, EFSA NDA Panel and important legal firms specialized in these matters. The potential of technologically based spin-off companies as an appropriate mechanism to proceed with further development and validation of the

discoveries ensuing specific and commercial targets has been considered within the Consortium (e.g., the foundation of a spin-off dedicated to the measurement of homeostatic robustness based on putative novel biomarkers identified within BIOCLAIMS). Necessary steps have been identified. However, it was identified that, at present, a consensus framework at EU level to facilitate the exploitation of joint results through the creation of a spin-off of this kind involving several partners from different European institutions is lacking, and would be desirable.

Achievements of BIOCLAIMS are in accordance with the ones expected as stated in the Project proposal:

- The characterization of metabolic robustness in front of different forms of stress (metabolic stress, oxidative stress, inflammatory stress);
- The development of novel, nutrigenomics-based biomarkers for characterizing healthy phenotypes and highly prevalent conditions of impaired homeostatic control;
- The provision of scientific evidence on effect of aging and gender on biomarkers of homeostatic control;
- The provision of scientific evidence on the effects of early priming on biomarkers of healthy aging;
- The development of biomarkers for mapping the intrinsic effects of bioactive food components present in the normal diet under conditions of homeostatic control covering a continuum ranging from normal to impaired;
- The development of harmonized European tools to allow data comparability across Europe and improve the biomarker concept in nutrition research.
- The provision of scientific evidence to help support the development of health claims on food.

BIOCLAIMS has produced nutrigenomics-based biomarkers and other biomarkers of health robustness of potential application to substantiate beneficial effects of nutrients or bioactive compounds in infants and adults leading to novel food claims on foods. Food industry has shown great interest in these results, and from this expressed interest the establishment of collaborations of BIOCLAIMS partners with industrial partners − at a different level, more exploitation-oriented and beyond the pre-competitive research financed by the BIOCLAIMS call − is expected. Candidate biomarkers have been tested for validation within BIOCLAIMS up to a reasonable level. They have been verified in different animal models and/or human studies. From here, the involvement of parts more directly interested in the development and exploitation of concrete biomarkers, on a case by case basis, is required.

Part 5. The address of the project public website, if applicable as well as relevant contact details

The BIOCLAIMS project web site can be reached from www.bioclaims.eu and www.bioclaims.org at http://bioclaims.uib.es/

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