COMMENTARY

The NuGO proof of principle study package: a collaborative research effort of the European Nutrigenomics Organisation

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Introduction

The European Nutrigenomics Organisation (NuGO; www.nugo.org) was established in 2004 and is funded within the Sixth Framework Programme of the European Commission as a network of excellence [4]. The core partners forming NuGO are 23 research units based at universities and research centers. The key objectives of

NuGO are: (a) the promotion of molecular nutrition research in Europe; (b) the development and promotion of mechanistic research in nutrition, food and health research by application of *omics* technologies; and (c) to develop joint research programs. The NuGO Proof of Principle Study package (NuGO PPS) started in 2007 and is one of the collaborative research initiatives within NuGO. The main goals of the NuGO PPS are to: (a) assess the advantages and the limitations of *omics* applications in nutritional studies; (b) prove that systems biology

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approaches can provide a better description of the health/disease status of an organism; (c) prove NuGO's ability as a network of excellence to analyze complex data sets obtained from a federated series of studies, performed at many partner sites, analyzed in different *omics* platforms in combination with statistics and bioinformatics in an integrative way; and finally (d) optimize the convergence in data sharing by implementing a data integration platform.

The organizational structure of the NuGO PPS comprises a core team formed by six leading scientists (C.A.D., R.M.E., M.G., R.K., M.M., B.v.O.) who defined the general outlines of the NuGO PPS, a coordinator team (H.D., I.R.-A.), three main centers where the experimental studies were performed and 12 centers where samples are analyzed in a close relationship between "wet lab" and "dry-lab" scientists (Fig. 1). Moreover, there is a study that links NuGO to another Framework 6 Integrated Project, LIPGENE (www.ucd.ie/lipgene), which is an integrated project on the theme of the metabolic syndrome.

The experimental research platform is based on three mouse feeding trials, PPS1, PPS2, PPS3, and a human volunteer trial referred to as the human PPS (both animal and human studies are approved by their respective local ethics committees). All studies are based on an energy challenge and the acute and chronic effects on substrate oxidation and inflammation status in addition to the long term alterations and stability of energy homeostasis. The mouse studies address the effects of time (PPS1) or metabolic challenges (PPS2) or dietary fat content (PPS3) on the development of insulin resistance and the metabolic consequences which are assessed by comprehensive phenotyping. Analysis takes into account the type of diet, the time-dependency of changes and organ-specificity of effects well as the ability of an organism to cope with

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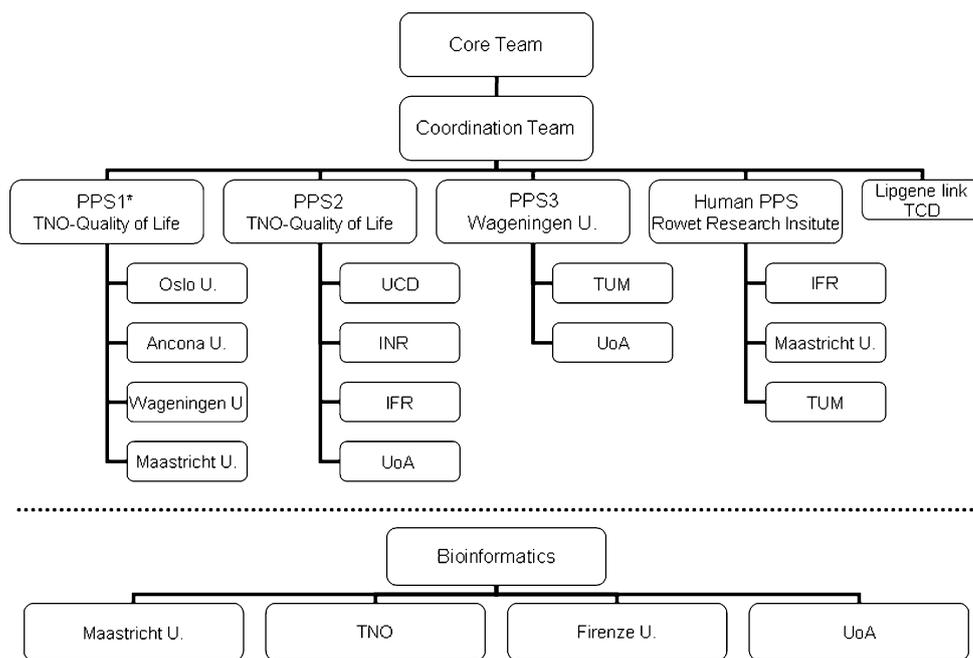University College Dublin, Dublin,
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metabolic dietary stress (bolus of glucose). The human study is designed to assess the biological intra- and inter-individual variation of a huge spectrum of molecular, metabolic and clinical parameters employing the different *omics* technologies and to assess the scale and patterns of alterations elicited by a simple nutritional intervention—a 36 h fasting period.

PPS1: development of insulin resistance on a high fat diet in ApoE*3Leiden mice

The ApoE*3Leiden transgenic mouse is highly susceptible to diet-induced hyperlipoproteinemia and has been established as a valid murine atherosclerosis models mimicking many hallmarks of human metabolism related to diabetes and atherosclerosis [8].In ApoE*3Leiden transgenic mice, the lipid metabolism is humanized (i.e., a more human-like lipoprotein profile). These humanized mice also can serve as a model of insulin resistance. The mouse study was designed and executed by T.K. and R.K., at TNO-Leiden (The Netherlands) including microarray analysis of organs relevant for insulin resistance (liver, adipose tissue, muscle). Parts of the study were donated to NuGO and biosamples are being further analyzed within the NuGO PPS1 research community. The prime goal of the study is to monitor, with high time-resolution, the development of diet-induced obesity (DIO) and insulin resistance. To do so, genome-wide transcriptomic datasets for the course of the development of insulin resistance in liver, white adipose tissue and muscle were generated. Within PPS1, the development of hepatic steatosis is analyzed which includes the development of a (subacute) inflammatory response. Insulin resistance was achieved in the ApoE*3Leiden mice by feeding animals a diet based on 45% fat by energy (beef tallow) over 12 weeks. Insulin resistance was established in the animals by performing a glucose tolerance test 3 day prior to sacrifice (and parallel hyperinsulinemic-euglycemic clamp analyses in separate mice). Tissue samples were collected at 0, 1, 6, 9 and 12 weeks (sacrifice of $n = 15$ animals per time point) and plasma samples were taken in each animal at regular intervals to be able to analyze intra-individual changes over time. Three different adipose tissue depots (mesenteric, abdominal subcutaneous and epididymal) are being analyzed by transcriptomics, histology and immunochemistry to assess the response of the different depots to insulin resistance and to obtain potential new biomarkers of adipose tissue insulin resistance. These analyses will be performed taking into consideration that the mesenteric adipose tissue can be markedly contaminated by pancreatic tissue in the proximal part of the mesenteric adipose depot [2]. Pancreatic contamination of the mesenteric adipose

tissue can be minimized by discarding the proximal part of the mesenterium located close to the pancreas. The liver is being analyzed by transcriptomics, targeted proteomics as well as histology and immunochemistry. In addition, inflammation markers (for review: [5, 6]) are monitored in liver and plasma to follow the progression of insulin resistance and hepatic steatosis. Moreover, collaborations have been established with other groups within NuGO to analyze the impact of this metabolic challenge on colonic tissues (G. Elliott and L. Lund, Institute of Food Research, Norwich, UK).

PPS2: evaluation of the acute whole-body response to glucose stress in C57BL/6J mice

PPS2 was designed to compare the whole-body response to a metabolic stressor (glucose bolus) between healthy and insulin resistant mice. Mice with an isogenic background (C57BL/6J mice) were fed diets for 12 weeks containing either 10 or 45% fat by energy (mainly palm oil) after 2 weeks of adaptation to laboratory conditions with a control diet. Plasma was collected throughout the 12 weeks of feeding to monitor the development of insulin resistance establishing that animals on the high fat diet had developed insulin resistance after 3 months. Healthy and insulin resistant animals were subjected to a glucose tolerance test at the end of the feeding trial followed by comprehensive phenotyping. The core of the study was performed at TNO (coordinated by R.K. and T.K.). Serum, liver and selected tissues like muscle and adipose tissue were removed 0 min, 40 min, 2, 18 and 48 h after the glucose challenge. Glucose

was injected via the i.p. route at 1 p.m. in all groups and animals had free access to feed during the challenge (no fasting). The liver will be analyzed by transcriptomics, and by proteomic and metabolomic approaches. Metabolomic analysis is also being performed in muscle, another tissue affected by insulin resistance, whilst serum is being analyzed by both proteomics and metabolomics. Moreover, the collaborations mentioned in PPS1 have been established here also to analyze the impact of this metabolic challenge on colonic tissues.

PPS3: dose-dependent effects of dietary fat on the organ-specific response in C57BL/6J mice

Prime aim of this study is to generate robust *omics* data sets for the effects of diets comprised of increasing fat contents on the organism. The core study was performed at Wageningen University (coordinated by M.M. and M.V.B.). C57BL/6J animals were fed diets with 10, 20, 30 and 45% energy from fat for 4 weeks (after 2 weeks adaptation to a control diet). Transcriptome analysis is being performed in liver, small intestine, adipose tissue and muscle on samples obtained after 0, 1 and 4 weeks of feeding. Moreover, proteomic analysis of liver and muscle tissue is being carried out on the same samples to enable direct comparison with the transcriptomic data. Pancreatic tissue is analyzed at the proteomic level due to its importance in insulin secretion. And in collaborations with other NuGO groups colonic (D. Haller and T. Werner, TUM) and brown adipose tissue (M. Klingenspor and T. Fromme, TUM) are also being analyzed. Since not only glucose and lipid metabolism but

also amino acid metabolism (5) are insulin-dependent and change during a decline of tissue insulin sensitivity, another aim of PPS3 is to assess whether changes in plasma amino acid levels or patterns can serve as biomarkers of insulin resistance. LC-MS/MS will be applied to quantify 45 amino acids and derivatives in plasma samples collected every second week from animals fed the 10 or the 45% energy from fat diets over the 12-week period. Another study arm embedded into PPS3 is comprised of a leptin challenge (coordinated by B.deR. and L.M.W.). In this study we perform transcriptomic analysis of the hypothalamus, proteomic analysis histochemical analysis of hepatic cells in mice fed 10 or 45% energy from fat for 1 or 4 weeks, with or without an acute leptin challenge. This will allow us to assess, at the tissue level, changes in leptin sensitivity and alterations that are central to satiety and metabolic control [1, 5]. Such changes will be related to plasma leptin and glucose levels.

Human PPS: an intervention study

The human PPS represents a continuation and extension of an established collaborative effort within NuGO based on the PlasmaPlus Study in which four centers validated and optimized proteome analysis from blood samples for biomarker discovery [3]. Main aims of the human PPS is to (1) assess the biological intra-and inter-individual variance of a huge spectrum of molecular, metabolic and clinical parameters employing the different *omics* technologies at baseline and after a 36 h fasting period, (2) identify common and unique changes in these parameters in response to fasting, and 3) determine the most comprehensive and sensitive *omics* approaches to detect and quantify the effects of a nutritional fasting challenge in humans. The human PPS enrolled ten healthy volunteers that were asked to come to the Human Nutrition Unit in Aberdeen on four different days of the week during four weeks after an overnight fast to provide a blood sample (for the isolation of plasma, platelets and PBMC), and a saliva and a morning urine sample to assess intra- and interindividual variations in baseline levels. On the fourth sampling day, volunteers were fasted for an additional 24 h (total of 36 h) and again plasma, saliva and urine samples as well as circulating blood cells were collected for transcriptome, proteome and metabolome analysis. The core study was performed at the University of Aberdeen (coordinated by B.deR. and S.J.D.). All samples are being submitted to comprehensive profiling employing all *omics* technologies. Biological variability and fasting responses are being monitored in plasma, platelets, peripheral blood mononuclear cell (PBMC), urine and saliva by proteomics based on 2D-gel based platforms coupled to mass spectrometry for

protein identification. Alterations in the PBMC transcriptome are being analyzed by employing the Affymetrix microarrays specifically designed for NuGO, and plasma and urine sample metabolite profiling is achieved based on NMR, GC-MS, GC-MS/MS and LC-MS/MS platforms comprising non-targeted and targeted approaches. Moreover, a panel of 89 hormones, cytokines and chemokines and metabolic factors was analyzed on a commercial targeted profiling platform (RulesBasedMedicine, www. rulesbasedmedicine.com). In this unique combination of nutrigenomics tools this study should provide the most comprehensive metabolic phenotyping possible in human volunteers with minimal invasive methods for obtaining appropriate biosamples.

Bioinformatic studies

All data generated in the four experimental studies are being collected and analyzed using a central data warehouse represented by the NuGO "black box system" also comprising a NuGO-wide intranet platform for data processing and analysis. A group of bioinformatic experts (coordinated by C.E) is responsible for the analysis of the data by different bioinformatic and biostatistics approaches. The studies are focused on the statistical modeling of the time-courses and organ-specific responses to the dietary challenges with a particular focus on the transcript profiling data sets. A second series of analysis uses the time course results and targets integration of transcriptomic, metabolomic and proteomic data by multivariate analyses and at the pathway level by using, for example, PathVisio [7]. In this issue Coort S. L. et al. and Baccini M. et al. describe some of the biostatistical and bioinformatic approaches applied to the different study sections with the NuGO PPS.

Standardization and integration

The NuGO PPS package is the outcome of a long process of discussions between the nutrigenomics experts in the network. Apart from the scientific innovation and excellence mentioned previously, a prime prerequisite was that all partners submitting proposals to join the PPS trial could collaborate within a virtual research infrastructure. All participants had to agree on the highest level of standardization of the methodologies used and on standardized data storage and data sharing policies. In fact, this study was intended also as a proof of principle for the established NuGO research infrastructure. The experimental designs of two of the three mouse studies were harmonized by defining animals, age, gender, diets, fasting periods and the

workflow in obtaining and processing the biosamples. Shared analytical platforms such as the NuGO Affymetrix gene chip, a common hybridization service as well as the stream-lined data analysis should ensure the highest degree of harmonized data generation and standardization. Also, the NuGO PPS profits from the infrastructure already created in the previous years within NuGO such as the array pipeline and the NuGO Black Box Project which is formed by a federated network of computer servers installed at all partners, allowing standardized storage, sharing and processing of all data generated within the PPS (Evelo et al. in press).

Conclusion

Here we present the NuGO Proof of Principle Study package to the science community. This effort represents one of the very few coordinated interdisciplinary and multicenter research initiatives in the nutrigenomics field. The aims of the studies are to (1) improve understand of the time-lines for the development of insulin resistance in different organs under feeding regimens based on high fat diets, (2) take the *omics* technologies to proof and (3) take NuGO to proof as a network that is generating an "added value" by sharing technology, data, knowledge and expertise. In this respect not only mice and human volunteers are challenged but also the entire science community under the NuGO (p)roof.

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