Chronotherapeutic lifestyle intervention for diabetes and obesity to reset the circadian rhythm and improve cardiometabolic risk in the European working population

Reporting

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Executive Summary:
Modern European lifestyles have dramatically changed the daily, circadian, rhythm of life. Physical activity, food intake and light exposure are no longer restricted to daytime hours, and sleep duration has been continuously reduced over the last century. These lifestyle changes affect psychomental health, sleep and fatigue, and – as more recent studies indicate – cardiometabolic health. EuRhythDia aimed to understand the importance of circadian regulation of metabolism in the development of cardiometabolic risk associated with obesity, type 2 diabetes and cardiovascular disease. To achieve this goal, data from pre-existing, large, population-based cohorts were combined with lifestyle intervention studies in two high-risk groups, night shift workers and first degree relatives of patients with type 2 diabetes, and animal experiments using knock-out mice and the zebra fish model. Potential novel targets for intervention were screened for small molecule modulators, and novel biomarker assays developed.

Data from the population-based Gutenberg Health Study showed that night shift workers have a higher prevalence of obesity and impaired glucose levels than non-night shift workers. In prospective follow-up analyses, genes previously identified as diabetes risk gene markers were associated with a higher incidence of type 2 diabetes in the night shift worker subpopulation, too.

However, although the finding of a difference in glucose metabolism between night shift workers and daytime workers was reproduced in our intervention studies, circadian-adjusted intervention with light therapy, exercise, or melatonin in night shift workers during a period of 12 weeks had no major influence on glucose metabolism. Melatonin intake was found to be safe and did not negatively affect cardiometabolic risk. Similarly, a placebo-controlled trial with melatonin in normoglycaemic first degree relatives of patients with type 2 diabetes revealed neither benefit nor harm with respect to glucose tolerance; However, melatonin induced small but significant changes in HDL cholesterol and coagulation markers which deserve further investigation.

Out of the list of potentially drugable clock genes, Rev-erb-alpha turned out to be of major interest. Experiments with Rev-erb-alpha knock-out mice revealed that this nuclear receptor is of high relevance for mediating the circadian influence on skeletal muscle metabolism. In other experiments, zebra fish were
proven to be a suitable model for studying gene expression under conditions of sleep disorders, modifications of the light-dark cycle, and metabolic challenges. Our data suggest that utmost care needs to be taken on the selection of housekeeping genes in this model, as light conditions have a major effect on the expression levels of all genes studied.

Agonists and antagonists of Rev-erb-alpha were identified in our small molecule screening programme. Although the hits were not suitable for further drug development due to lack of selectivity, in vivo proof of principle studies demonstrated that Rev-erb-alpha is a highly promising target for intervention in the treatment and prevention of cardiometabolic diseases. Inhibitors of DDAH and of AGAT, two genes involved in the metabolism of biomarkers associated with cardiometabolic risk, were also identified that can now be characterized more thoroughly and followed up for their therapeutic potentials.

In summary, EuRhythDia gathered significant progress in our understanding of the circadian influence on glucose metabolism and cardiometabolic diseases. However, the project also demonstrated that single, short-term circadian-adjusted interventions do not suffice to improve glucose tolerance in apparently healthy individuals at high risk of developing diabetes. This finding underscores the complexity of glucose control in human subjects, and reinforces the need for multi-faceted and early approaches to the prevention of type 2 diabetes mellitus.

Project Context and Objectives:
The Setting: Type 2 Diabetes and Obesity, Lifestyle, and Circadian Rhythms in Europe

Rising rates of obesity and type 2 diabetes with associated morbidity and mortality make this one of the major health issues in the European Union in the 21st century, which already accounts for EUR 80 billion, representing 10% of total healthcare expenditure in Europe. Epidemiological evidence indicates that obesity-related insulin resistance predates the development of frank type 2 diabetes by up to 20 years, but that during this important period, the risk of cardiovascular disease already begins to emerge. As individuals cycle from euglycaemic insulin resistance to beta cell failure and frank hyperglycaemia, further increases in cardiovascular risk occur and, following the emergence of type 2 diabetes, the risk of microvascular complications develops. This understanding is important because the long prodromal phase before diabetes appears provides opportunities for lifestyle interventions to ameliorate disease progression. In addition, this model supports the view that clustering of cardiometabolic risk prior to the development of diabetes increases cardiovascular disease, whilst hyperglycaemia, when diabetes appears, further increases cardiovascular risk and additionally increases microvascular complications. The direct and indirect costs associated with the morbidity arising from this disease progression will have a major impact on the national budgets of EU member states.

Whilst progress has been made in the pharmacological treatment of type 2 diabetes mellitus in recent years, no convincing specific strategies have been proposed for the prevention of this disease, except general recommendations regarding weight loss, regular exercise, and a healthy lifestyle. It has always been assumed that these approaches are effective because they influence energy balance in whatever manner they are applied. However, exciting recent data from experimental and clinical research strongly supports the view that weight gain, for example, disrupts the endogenous clock and that this disruption puts metabolism out of time with our energy needs creating a strong determinant of type 2 diabetes risk. This suggests that lifestyle intervention might benefit from consideration of the impact of the timing of application during the day.

In murine studies genetic disruptions in circadian clock genes lead to the development of a phenotype similar to human type 2 diabetes/obesity. Although there are no studies in human subjects that causally relate clock genes to the development of type 2 diabetes, members of our consortium have reported that
variation in Clock genotype relates to the metabolic syndrome in man, and epidemiological data has shown that shift workers – who are known to experience disrupted circadian rhythms – are at increased risk of developing type 2 diabetes mellitus.

With no consolidated data on humans integrating genetic, biochemical, and clinical observations, there is a research gap in our understanding of the molecular and causal links between lifestyle, circadian rhythm disruption, and diabetes / obesity in man. The EuRhythDia consortium aimed to fill this gap in susceptible target groups. These span from night shift workers (an extreme of lifestyle associated with disrupted sleep/wake rhythms) to rotating shift workers, from juveniles who voluntarily stay up late, thereby adopt a lifestyle disrupting their circadian rhythms, to elderly people who have a high prevalence of sleeping disorders, from menopausal women affected by sleep disorders and disturbed circadian rhythms to an increasing number of long-distance travellers suffering from jet lag. This long list underscores the fact that a large and increasing part of the European population is affected by lifestyle conditions associated with disruptions of circadian rhythms, increasing their risk of obesity and cardiometabolic disease.

The EuRhythDia consortium’s research efforts utilized data from existing prospective cohorts with the aim of dissecting molecular effects and the causality of circadian disruption in humans; it spanned existing genetically engineered animal models, high throughput screening methods, and ‘omics’ approaches. In addition, our group went far beyond this by recruiting small, intensely phenotyped cohorts of high-risk populations in which we applied novel lifestyle interventions to ameliorate risk.

The Background: Insulin Sensitivity – A Physiological Phenomenon Controlled by Circadian and Circannual Oscillators

Animals that overwinter put on weight in the autumn and develop severe insulin resistance, a process that is gradually reversed through the winter months as the animal loses weight, leading to the emergence in spring of a thin insulin-sensitive phenotype. By this means the overwintering animal cycles metabolism and weight gain according to seasonal needs. Modern man, in contrast, tends to chronically gain weight irrespective of seasonal requirements. Therefore, what in most animals is a physiological response that reduces energy expenditure at appropriate times to allow the animal to survive prolonged periods of absent food intake, in man becomes pathological through prolonged exposure, which is a paradigm for many of the modern diseases in which exposure is a crucial element. Similar physiological regulation of metabolic processes takes place on a circadian basis, maximising nutrient and energy supply to peripheral tissues when the organism is active, and reducing energy uptake during sleep. In humans, the morning rise in cortisol and other hormonal systems upregulate glucose supply to prepare the human body (specifically skeletal muscles) for the day. During night shift work, these physiological rhythms which have been imprinted during thousands of years are maintained, resulting in dissociation between optimal energy supply (day) and maximal energy need (night). The night shift worker sleeps during the day and takes the major part of his meals during night time, when the organism is relatively insulin-resistant. In patients with type 2 diabetes, the endocrine-mediated circadian regulation of energy supply and energy expenditure may be dysbalanced because the interplay between the various involved hormones is disrupted, but a detailed molecular understanding of the underlying processes is lacking. Systematic analyses of lifestyle-induced changes in circadian rhythms and their influence on metabolism may help to better understand the underlying biology, and to develop better targeted strategies for diagnosis, prevention, and treatment.

The Problem: Shift Work Leads to Disruption of Circadian Rhythms and Promotes Diabetes, Obesity and Cardiovascular Disease

Modern European lifestyle has dramatically changed the daily rhythm of life. Physical activity, food intake and light exposure are no longer restricted to daytime hours, and sleep duration has been continuously
reduced over the last century. Technical and economic demands fuel the necessity of work outside normal working hours. European statistics reveal that 12% of the working population in the EU15 states and 23% in the new EU member states maintain a shift work schedule, whilst 4.4% and 5.4%, respectively, work at night. Moreover, the Survey among Young People Ages 15-30 in the European Union exemplifies that an increasing number of adolescents develop unhealthy lifestyles by staying up late, spending less time exercising, and taking untimely meals.

A recent prospective study which included 402 night-shift workers and 336 daytime workers who were followed for a median 4 years uncovered a 5-fold elevated risk of developing type 2 diabetes/obesity in night-shift workers. Another prospective study comprising a cohort of 1,529 workers (309 rotating shift workers) found a 77% higher incidence of the metabolic syndrome in shift workers during 6.6 years of follow-up. Even before forty years of age, shift workers display increased intima media thickness and higher risk for carotid plaques compared with non-shift workers, indicating accelerated atherosclerotic processes. This is interesting and highly relevant in societal and economic contexts, as cardiovascular events are the major determinant of reduced life expectancy in patients with type 2 diabetes and obesity.

The development of occlusive cardiovascular disease is characterised by complex interactions between inflammation, procoagulant mechanisms, vascular dysfunction and remodelling. Over time this leads to the formation of atherosclerotic lesions, plaque instability and, ultimately, vascular occlusion mediated by the formation of a platelet-rich fibrin clot. Insulin-resistant type 2 diabetes patients cluster inflammatory atherothrombotic risk which predisposes to early and more severe arterial thrombotic disease. Accumulating evidence suggests that adapting shift work schedules to biologic rhythmicity improves sleep, occupational performance, and markers of metabolic and cardiovascular disease risk. For example, clockwise rotating shift work has been demonstrated to be better tolerated than counter-clockwise rotational shift work in a controlled study with 45 volunteer police men. A population-based case-control study comparing 2,006 cases with first-time myocardial infarction with 2,642 controls found that shift work increased myocardial infarction risk by 30-50% across age groups; however, women between 45-55 years of age had a 3-fold increase in risk. Whilst other studies have corroborated the observation of gender differences in the sensitivity to the negative effects of (night) shift work, this has never been studied in population-based prospective cohorts.

The Objectives of EuRhythDia

EuRhythDia aimed to develop understanding of the importance of circadian regulation of metabolism in normal health, and in the development of cardiometabolic risk associated with obesity, type 2 diabetes and cardiovascular disease. In order to achieve this, the consortium’s research efforts combined data from an existing large prospective cohort with that from new interventional cohorts, supplemented by existing genetically engineered animal models, high throughput screening methods, and ‘omics’ technologies to try to identify novel biomarkers of circadian disruption and characterise the effects of novel lifestyle interventions on cardiometabolic risk.

Specific objectives:

• Analyse the influence of circadian rhythm disruption on the activity patterns of clock genes, expression of nuclear receptors, and cardiometabolic risk. This was carried out in a pre-existing, well phenotyped population-representative cohort (Gutenberg Health Study) and in a novel, prospective night-shift workers cohort (Italian Nurses Study). These cohorts were followed for the duration of this project and enabled us to assess as a primary outcome the prognostic value of novel biomarkers for the development of obesity and type 2 diabetes.
• Investigate the effects of randomized, controlled chronotherapeutic lifestyle and pharmacological
interventions (exercise, light therapy, and melatonin) on markers of central and peripheral circadian rhythms and of cardiometabolic function. This was carried out in newly designed and intensely phenotyped cohorts of night shift workers and of first-degree relatives of patients with type 2 diabetes. Night shift workers were subjected to chronotherapeutic lifestyle interventions to reset circadian clocks. The effects of short term intervention on circadian disruption, glucose metabolism and insulin resistance were assessed after 12 weeks. Intensive phenotyping included documentation of sleep quality, quality of life, and chronotype by validated questionnaires, and actigraphy studies to quantify light exposure and activity periods during the day. This part of the studies also served to compare the relative effectiveness of different chronotherapeutic lifestyle interventions with each other. Data gathered in night shift workers were transferred to clinically healthy subjects with a high risk of developing type 2 diabetes. For this, first degree relatives of patients with type 2 diabetes were recruited and melatonin administration was utilized to reset their circadian clocks in a double-blind, placebo-controlled trial. After long-term intervention (6 months) the effects of the intervention on biomarkers of circadian disruption and the effects on glucose metabolism, insulin resistance and cardiometabolic risk were assessed.

- Dissect the molecular mechanisms linking circadian clock genes with glucose metabolism and metabolic syndrome phenotype in animal studies. For this, genes amenable to modulation by small molecules were selected, and specific genetically engineered mouse models were available in the consortium. We also created a high-throughput, economical animal model in zebrafish using existing breeding and phenotyping facilities for fast screening of effects of environmental and genetic factors on metabolism.
- Apply novel diagnostic assay development and drug discovery approaches, integrating novel targets identified in the consortium.
- Disseminate our findings to the European public, helping to improve prevention of diabetes and obesity in the European working population. Beyond existing strong links of academic consortium partners into the scientific community, partnering with International Diabetes Federation (IDF) Europe, the European diabetes patients’ main organisation, ensured transfer of complex scientific contexts into language communicable to shift workers, patients with diabetes and their relatives, and the general public.

Project Results:
According to the EuRhythDia work plan, the main scientific results are grouped into the results obtained from:

- The pre-existing population-based cohorts;
- The intervention studies in night shift workers and in first degree relatives of patients with type 2 diabetes;
- The animal experiments;
- The drug discovery efforts.

Results from the population-based cohorts

EuRhythDia had access to the large, population-based cohort of the Gutenberg Health Study. Out of a total of 7,856 individuals, 677 (8.6%) were active night shift workers working a median 5 nights per month. The basic result of the cross-sectional analysis comparing night shift workers with non-night shift workers were significant phenotypical differences, with a major focus on the prevalence of obesity and the metabolic syndrome, a higher mean fasting serum glucose and HbA1c, and significant differences in some small molecule biomarkers. These results have been published (Jankowiak et al. Current and cumulative
night shift work and subclinical atherosclerosis: results of the Gutenberg Health Study. Int Arch Occup Environ Health 2016; 89: 1169-82).

Subsequently, a prospective analysis was performed into which 13,745 individuals were included. 24.3% of this population were night shift workers, working a median 5 nights per month. An integrated analysis comprising incident diabetes and pre-diabetes, gene expression profiles, and biomarkers analyses was performed to evaluate the relative contribution of night shift work versus the genetic predisposition of individuals to diabetes on the incidence of the disease. These results, which have been obtained just shortly before the end of the EuRhythDia project period, are currently being analysed in-depth and prepared for scientific publication.

In addition, a clinical study published in Pietroiusti et al. in 2010 had compared night shift workers and non-night shift workers regarding the incidence of the metabolic syndrome. This study had revealed an about 5-fold higher incidence of the metabolic syndrome in night shift workers. We called back all participants of this study into the study centre for additional blood sampling. The objective of this study was to create a biobank from these subjects to store serum, plasma, peripheral blood monocytes, DNA, and clinical data to study clock genes and circadian rhythm biomarkers through metabolomics, epigenetics and quantitative real time PCR. After arrival in the study centre at 7.30-8.30 a.m. fasting blood samples were collected (for biochemical/cardiometabolic biomarkers, metabolomics analysis, for separation of PBMCs and DNA/RNA extraction). Patient history, anthropometrics, and demographics were recorded, and participants were asked to fill in questionnaires [FINDRISK score, the short questionnaire to assess health enhancing physical activity (SQUASH) and Pittsburgh Sleep Quality Index (PSQI)].

Prospective follow-up analysis of the study participants was performed after a mean follow-up time of 37 months. Data of the cross-sectional plus the prospective analysis of this cohort confirmed the higher risk of impaired glucose tolerance in night shift workers as compared to daytime workers. Interestingly, ex night shift workers appeared similar to active night shift workers, suggesting that metabolic disturbance induced by night shift work persist after ceasing this job activity. Detailed results of this study, including metabolomic analyses and gene expression results, are currently being prepared for publication.

Results from the intervention studies in night shift workers and in first degree relatives of patients with type 2 diabetes

Within the framework of EuRhythDia, three intervention studies were conducted. These intervention studies were targeted to address individuals with a high risk of type 2 diabetes mellitus and disruptions of the circadian clock. Based on previous publications and observations by members of the EuRhythDia consortium, night shift workers and first degree relatives of patients with type 2 diabetes were selected as target groups. Two intervention studies addressed night shift workers, using several different types of lifestyle interventions, and a third trial comprised first degree relatives.

A. Night shift workers’ studies
Four centres (Hamburg, Aachen, Rome, and Salzburg) participated in recruiting night shift workers of both male and female genders. The objective was to perform three different short-term chronotherapeutic...
lifestyle interventions, all with a duration of 12 weeks, and a second re-analysis after additional 12 weeks of wash-out, according to identical standardized protocols. With this study protocol, we aimed to prove causal relationship between modulations of circadian rhythm and glucose metabolism.

The interventions were: Light therapy (one cumulative hour of daylight therapy during the first half of the night shifts), exercise (30 min of high intensity training within a time frame of 2 hours before each night shift), and melatonin intake (2 mg of melatonin sustained-release before bedtime [i.e. at night on regular days, in the morning hours after a night shift]). Data were compared to a no-intervention control group (light therapy, exercise; randomized, unblinded study design) and to a placebo group (melatonin administration; randomized, double-blind, placebo-controlled study design). This was necessary for regulatory reasons: Intervention with light therapy and exercise was deemed a non-drug trial by the responsible Ethics Committee, and was performed in one protocol with the no-intervention control group in an unblinded fashion; melatonin treatment was deemed to be a drug trial and was performed as a multicentre, double-blind, randomized, placebo-controlled trial. In order to comply with European drug legislation, melatonin was administered in the form of Circadin® 2 mg sustained-release tablets, a drug formulation approved in the European Union.

A subgroup of 12 study participants from each of the intervention and control groups plus a group of apparently healthy, non-night shift working human subjects were submitted to intensive phenotyping, which included a 24-hour in-patient stay during which 3-hourly blood sampling and processing was performed. Blood samples were subjected to melatonin and cortisol analyses, as well as biomarker and metabolomics analyses. Beyond this, all study participants filled questionnaires and performed oral glucose tolerance tests at each study visit. The participants in the exercise intervention group and in the control group performed spiroergometries at each study visit, in order to control for a possible endurance training effect, which would have affected glucose control independently of circadian regulation of metabolism.

During each of the study visits (baseline, post intervention [12 weeks], and post wash-out [24 weeks]), identical procedures were performed: Collection of fasting blood samples, recording of patient history, anthropometrics, and demographics, oral glucose tolerance testing, and questionnaires (sleep, Findrisk, and chronotype). The primary endpoint was defined to be the change in the area under the curve of blood glucose derived from the oral glucose tolerance test between baseline and 12 weeks of intervention. Secondary endpoints related to insulin levels, indices of insulin resistance, HbA1c, and biomarkers.

A huge effort was needed from all recruiting centres to complete the study; results were thus obtained only shortly before the end of the project and have not yet been published at the time of submission of this report. Exciting results were found for some of the interventions that shed light on the causality of the relationship between disturbances of the circadian clock and glucose metabolism. As scientific, peer-reviewed publications are being prepared by EuRhythDia co-workers presently, detailed results cannot yet be presented here but will be available shortly through the publications.

B. First degree relatives’ trial
A third intervention study comprised first degree relatives of patients with type 2 diabetes mellitus. Individuals were included in the study if they had at least one first degree relative with an established
diagnosis of type 2 diabetes mellitus and were 18-75 years old. The participants were healthy subjects with no clinical symptoms or signs of infection or systemic disease, including type 2 diabetes mellitus, renal and liver impairment. Pregnant/breastfeeding females were excluded from the study. A total of 4,000 patients with type 2 diabetes were screened and 340 relatives of patients could be contacted.

A total of 75 patients willing to participate were subsequently randomised in a 1:1 random allocation to either 6 months of melatonin therapy (2mg prolonged-release melatonin tablet Circadin®) (n=37) or placebo (n=38). Circadin® or placebo were taken once daily in the evening 2h before sleep. The study team and patients were blinded to the randomization. Fasting blood samples were collected at 4 study visits, with the first taking place at randomisation (baseline or visit 1). Visit 2 and 3 were scheduled 3 and 6 months after randomisation respectively. All participants finished randomised treatment at visit 3. The final set of blood samples was taken at visit 4.

As this trial was planned to be the final intervention within the EuRhythDia project, results were obtained only shortly before the end of the project and have not yet been published at the time of submission of this report. A scientific, peer-reviewed publication is being prepared by EuRhythDia co-workers presently; therefore, detailed results cannot yet be presented here but will be available shortly through the publication.

Results from the animal experiments

Animal experiments were planned in two animal models which offered promising opportunities to study the effects of lifestyle interventions on glucose metabolism. Firstly, mouse models lacking Rev-erb-alpha (Rev-erb-alpha ko mouse) or ROR-alpha (ROR-alpha ko mouse) were available within the EuRhythDia consortium. Rev-erb-alpha and -beta are among the genes involved in the transduction of the central circadian clock onto the metabolic pathways, and were therefore identified a priori by the EuRhythDia consortium to be among the genes of primary interest. Secondly, the zebra fish model was established and validated as an animal model suitable for high-throughput investigation of the effects of lifestyle factors on gene expression.

Experiments in Rev-erb-alpha ko mice

The two nuclear receptors, Rev-erb-alpha and ROR-alpha, have been identified as clock components and as such play an important role in the biological clock function maintenance. Mounting evidence indicate that both Rev-erb-alpha and ROR-alpha also modulate metabolism in a circadian manner, indicating they may represent a bridge connecting metabolism and other clock components. Interestingly, both are drugable receptors which have been shown to respond to synthetic ligands and thus represent interesting targets for the treatment of clock disorder-related metabolic diseases. The studies performed within EuRhythDia were aimed at determining the role of Rev-erb-alpha and ROR-alpha in metabolism with a special emphasis on their function in metabolic tissues such as muscle. Indeed, muscle is responsible for the uptake of 80% of blood glucose and therefore plays a major role in the development of insulin resistance and diabetes. In addition, exercise has been shown, together with time-restricted feeding, as one of the most efficient clock synchronizers (‘zeitgebers’) and could prove useful to reset peripheral clocks in patients with metabolic diseases, supposedly through its action on clock proteins. As several
papers have been published which report the role of ROR-alpha in skeletal muscle metabolism since the writing of the EuRhythDia project, our studies focussed on the role of the clock component Rev-erb-alpha in cardio-metabolic diseases.

EuRhythDia researchers have previously shown that Rev-erb-alpha expression is increased upon exercise to promote mitochondrial biogenesis and function and induce muscle oxidative capacity. This leads to increased exercise capacity. These results have been published in Nature Medicine (Woldt et al., Rev-erb-alpha modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy. Nature Medicine 2013 Aug;19(8):1039-46). In addition, we examined the metabolic phenotype of the Rev-erb-alpha ko mice in response to a high fat diet. Our results show that the KO mice gain more weight than the WT mice, suggesting that Rev-erb-alpha controls energy balance. We went further and performed glucose tolerance tests using whole-body Rev-erb-alpha -deficient mice fed either a chow or a high-fat diet which revealed impaired glucose tolerance of these mice compared to their wild-type littermates on both diets. Altogether these data confirm that Rev-erb-alpha can be used as a therapeutic target to improve skeletal muscle mitochondrial function and glucose handling.

When investigating the metabolic adaptation of Rev-erb-alpha mice in response to exercise, we found that Rev-erb-alpha -deficient mice display decreased exercise capacity. This, together with the results of our metabolic studies, suggests that in the absence of Rev-erb-alpha, time cues normally provided by exercise may be less efficient at resynchronising the clock, thereby leading to altered skeletal muscle function.

In addition, we have used a standard procedure to entrain peripheral clock to new time cues. To this aim, mice were maintained in a normal 12:12 light:dark cycle and fed a normal chow, but food access was restricted to the resting (light) period. Using this paradigm, we were able to reproduce the published effects of tRF (time restricted feeding), namely inversion of clock gene expression in liver from wild-type control mice. For instance, Rev-erb-alpha expression, which is high in mice fed ad libitum, was depressed when mice were fed exclusively during the light period.

The aforementioned data suggested that Rev-erb-alpha might be involved in the development of diet-induced atherosclerosis. We therefore determined whether Rev-erb-alpha influences atherosclerosis development in vivo in mice fed either a regular chow or a cholesterol-enriched diet (western diet, WD) for 6 or 12 weeks. Rev-erb-alpha ko mice were bred into an LDLr-/- genetic background that is prone to the development of the disease. Our data showed that Rev-erb-alpha deficiency led to altered lipid metabolism and increased inflammation, leading to the development of atherosclerosis.

Further, as yet unpublished experiments pointed to the possible mechanisms of action of this effect of Rev-erb-alpha on atherosclerosis development. These results will be disclosed to the public in a peer-reviewed scientific publication which is currently under preparation.

Experiments in the zebrafish model
When establishing an animal model in the laboratory, the natural behaviour of the species should be carefully considered. Zebrafish behaviour is dominated by light: in darkness, the motor activity is minimal, while it can be easily activated by light. Zebrafish larvae move in sharp, millisecond bouts flanked with
periods of immobility throughout the light period. These stereotyped movements/ fixed action patterns are well characterized, easily distinguished and the underlying neuronal circuits well known. Also in darkness, the mobility pattern is similar, but the overall mobility is decreased and the immobility periods are longer.

The question is: are the larvae “sleeping” in darkness or are they simply immobile, because as visual hunters they are unable to prey? We tried to answer this question using a fast (1000 frames per second) camera to record the phases of the startle response, which is a stereotypical behaviour characterized by two parts: short latency C-turn (SLC) 0-15 ms and a long latency C-turn (LLC) 15-40 ms. The SLC is a fast reflex executed by three pairs of reticulospinal neurons, including the Mauthner cells, while the LLC involves also central processing. The reflex was induced by low electric voltage.

To assess possible sleep homeostasis in the larvae we used forced mobility induced by continuous water flow. The natural response of the larvae is to swim against the flow. We regarded this as a more natural way of keeping the larvae awake than e.g. electric stimulation. We used 7-14 dpf larvae that swim 6 h against the flow (30-40 ml/min), the control group was exposed to a minimal flow of 1ml/min. The swimming took place either between 23-5 h in the dark or during the day at 10-16 h or at 16-22 h in either light or dark. The larvae were tested by short electric stimulation (5V) immediately after the swimming period under the same lighting condition as they were during swimming. The stimulations were repeated at 2 min intervals for 90 acquisitions. The number of larvae that responded by performing a LLC was decreased after swimming in the dark.

Based on this series of experiments we conclude that zebra fish larvae show decreased reactivity after a prolonged period of forced motor activity in dark, which may be a sign of sleep homeostasis.

Subsequently, we performed a genome-wide expression assay analysis in order to identify the relevant transcripts and pathways that are affected by sleep restriction, the light/circadian rhythm and food manipulations in zebra fish. Two weeks old wild type zebrafish larvae were raised for seven days in normal light conditions (LD 14/10), and then seven days in experimental lighting conditions: LL or LD 4.5/4.5 and LD 14/10. Either normal fish food or high fat food was served in each condition. After two weeks, larvae were sleep deprived (SD) for 9-12 hours using the forced-swim-method. Control larvae were kept in the same conditions but without forced swimming.

Using more than 55,000 gene transcripts in the microarray, pathway analysis revealed overrepresented and underrepresented gene groups relating to immunological responses and metabolic processes. These data thus confirmed results from mammals, including humans, that sleep restriction activates immunological responses and down-regulates metabolic processes.

From our experiments we conclude that zebra fish is an extremely light sensitive species, and surprisingly this light sensitivity overrides normal physiological regulation, as demonstrated by increased expression of house-keeping genes in both constant light and in desynchronized light condition. The effect of light is so massive that it appears to mask the possible effects of other handlings (sleep deprivation and food quality). Conclusions are diluted by the fact that the induction of house-keeping genes prevented their usage as means for normalization in PCR measurements for other conditions but normal light/dark cycle.

Results from the drug discovery efforts
Drug discovery efforts using a high throughput small molecule compound screening programme were graded into two phases, one consisting of pre-selected targets defined at the beginning of the project period, and the second consisting of targets emerging during the project duration. Diagnostic assays were developed in parallel to target screening.

Compound screening was performed for three targets, with one target addressed by potential activators and inhibitors. The targets were DDAH (dimethylarginine dimethylaminohydrolase), an enzyme involved in the metabolism of ADMA, which is a mediator linking cardiovascular risk and diabetes according to published studies. A high throughput screening assay was developed and validated, and screening was performed using a large small molecule library. Screening discovered potential novel DDAH inhibitors which are currently under further investigation. Simultaneously, a dried blood test for ADMA was developed as a ubiquitously available, easily feasible biomarker assay addressing the same pathway.

The second target, AGAT (L-alanine:glycine amindinohytransferase), is an enzyme involved in the biosynthesis of creatine and homoarginine, i.e. involved in energy metabolism. In addition, homoarginine is considered to be a risk marker for cardiovascular disease and has been linked to diabetes as well. Although a few methods for monitoring AGAT activity have been reported (radioactive assays, Western blot analysis, GC/MS measurements and colorimetric assays) these are often not suitable for screening a large number of compounds. We have developed a supramolecular tandem enzyme assay as a simple, inexpensive and label-free fluorescence-based method to continuously monitor AGAT activity (Nilam et al. A label-free continuous fluorescence-based assay for monitoring ornithine decarboxylase activity with a synthetic putrescine receptor. SLAS Discov. 2017). In parallel, a homoarginine diagnostic assay was developed which allows to quantify homoarginine as a biomarker, e.g. in drug development studies.

As the third target, Rev-erb-alpha was chosen based upon our animal experimental data described above. A sophisticated high throughput screening assay was developed which was then used for compound screening of our small molecule library. Screening revealed a series of hits that were subjected to further profiling, producing a number of possible agonists and antagonists of this target. Further evaluation of these compounds led to the identification of three compounds that yielded reliable and reproducible results. These compounds are undergoing further evaluation of their specificity and potency.

Potential Impact:
Based upon its unique concept, EuRhythDia has undertaken a huge research effort to close the research gap in the understanding of the link between circadian rhythm disruption and type 2 diabetes / obesity. EuRhythDia was designed to not only to show associations, but to prove causal relationships between circadian-targeted lifestyle interventions and to improve the prevention of type 2 diabetes and obesity, specifically in shift workers and in first degree relatives at risk. This is of great importance, as many studies have in the past demonstrated statistical associations between biomarkers and disease events even from large prospective cohorts which, in subsequent interventional studies, have failed to show a causal relationship with each other.

EuRhythDia has taken this experience up and combined data analysis from large, prospective cohorts (associations) with interventional cohorts aiming at reversing circadian rhythm disruption (proof of cause-effect relationship), plus additional animal experiments dissecting mechanisms, pathways, and genes involved. Part of the results of EuRhythDia have already been published in peer-reviewed scientific journals. Another part of the project results is being prepared for publication in the near future. Thereby, a large spreading of the scientific data gathered and the experiences won, mainly within the intervention
studies, will be made available for the scientific community and will help to guide future research.

People with diabetes have more outpatient visits, use more medications, have a higher probability of being hospitalized, and are more likely to require emergency and long-term care than people without the disease. People with diabetes, on average, spend 2.5 times more on medical care than people without the condition. This goes along with a loss of quality of life of both the patients and their relatives and bears enormous social costs.

By investigating the impact of targeted prevention measures EuRhythDia significantly contributed to the efforts of reducing the social cost of diabetes type 2 / obesity resulting out of circadian clock disruption. In particular, the results of the project may positively impact the following social groups: shift workers, juveniles who voluntarily stay up late and thereby adopt a lifestyle disrupting their circadian rhythms, elderly people who have a high prevalence of sleeping disorders, women in menopause who are often affected by sleep disorders and disturbed circadian rhythms, and long-distance travellers suffering from jet lag. With the dissemination strategy that EuRhythDia has adopted through International Diabetes Federation – Europe (IDF Europe) material spreading the information on EuRhythDia results has been made available to a large public of affected individuals within the target groups mentioned above.

In the European region in 2010, diabetes costs approximately EUR 80 billion annually, representing 10% of all health expenditures, and corresponding to some EUR 1,500 per patient on average. Currently, some EU countries (Austria, France, Sweden and the Netherlands) spend more than EUR 3,000 per person. This means an enormous burden to national healthcare systems across Europe, and European societies have to bear these costs. Disburdening the national healthcare systems by effective prevention measures, huge resources could be reallocated within the national budgets. European societies will benefit from this, e.g. by reduced direct costs associated with medical care for patients with type 2 diabetes, reduced prevalences of ensuing micro- and macrovascular complications including their enormous cost, reduced indirect costs related to absenteeism from work and lost productivity by early retirement. EuRhythDia results have pointed towards novel targets for therapeutic intervention and towards novel biomarker combinations that may help to better identify apparently healthy individuals who are at a high risk of developing type 2 diabetes mellitus. In addition, EuRhythDia results strongly suggest that efforts for disease prevention need to set on very early, as glucose metabolism is highly complex and regulated on several levels. Any single interventive measure may thus fail to show sufficient benefit for disease prevention.

The estimated 18% of the EU workforce who are shift workers have been identified as a high risk group for the early onset of type 2 diabetes, obesity and cardiovascular diseases due to lifestyle-related disturbance of circadian rhythms. An even higher percentage of the EU workforce is also affected by lifestyle-related disturbance of circadian rhythms, but to a lesser extent. Demands of the EU economy in specific industrial sectors (e.g. healthcare, hospitality, logistics and transportation) mean that occupational demands cannot easily be changed to mitigate these lifestyle-related risks of disturbance of circadian rhythms. In a percentage of cases, individuals will prematurely and permanently be removed from the workforce due to incapacity and ill health. Trends across the EU show increasing levels of incapacity to work due to medical conditions, which increases national healthcare, welfare and third sector costs, as well as reducing tax revenue from these workers. Unemployment due to incapacity and chronic illness not only removes these
individuals prematurely from productive employment, but also occupies productive workers necessary for their long-term chronic care, with a direct opportunity cost to the economic potential of the EU.

EuRhythDia investigators experienced an extremely high level of attention for the health problems addressed in this project and the potential solutions offered amongst shift workers themselves, their labour union representatives, and their employers. This attention span from hospital personnel (night shift nurses and their representatives) to harbour workers, police officers and firefighters and bus and taxi drivers – thereby representing the job spectrum comprised in the EuRhythDia shift workers studies. It is interesting to note in this context, however, that not every intervention may be suitable for every job characteristics (e.g. a bus driver cannot have a light therapy device on board while driving at night; a harbour worker performing hard physical work throughout the night cannot exercise strenuously for 30 min before his shift); nonetheless, the spectrum of lifestyle interventions studied within EuRhythDia allowed to cover all of these professionals’ needs.

Interestingly enough, beyond general interest of stakeholders, several employers whose workers participated in EuRhythDia intervention studies offered extra paid leave to their workers in order to facilitate their participation in the time-consuming intensive phenotyping procedures. This observation holds the promise that recommendations ensuing from EuRhythDia studies – such as they have been prepared as part of the consortium’s dissemination measures and are available through IDF Europe – will be well received in the EU labour market.

Onset of type 2 diabetes, obesity and cardiovascular disease in individual workers has a series of economic impacts on public, private and third sector employers across the EU. The high incidence of cardiovascular disease amongst patients with frank type 2 diabetes mellitus is the major factor increasing morbidity and mortality and the cost associated with it. The cumulative effects of individual employee’s reduced work effectiveness, increased absenteeism, and subsequently increased staff turnover, each due to decreasing health, all result in increased disruption with related costs, leading to reduced productivity and profitability for employers. Conversely, effectively targeting this high risk group within the EU workforce through the application of a series of mitigation measures is expected to reduce the impact of lifestyle-related disturbance of circadian rhythms, both in the short and longer term. Therefore, reduced risk or even prevention of early onset of type 2 diabetes, obesity and cardiovascular disease will contribute positively to employee contributions. Mitigation of risk will be more cost-effective than treatment of symptoms, and preventative measures are more cost effective than curative measures for EU Member States’ healthcare and welfare budgets. Thus, even small effects of lifestyle intervention measures on biomarkers of cardiovascular risk can turn out to have a huge socioeconomic impact if followed consequently in a large population.

Dissemination procedures that were taken by EuRhythDia consortium members throughout the project duration comprised a wide variety of different activities. Besides a number of scientific publications relating to work in the various EuRhythDia work packages, an enormous number of oral presentations given throughout scientific conferences and workshops throughout the world and specific measures like the distribution of flyers, newsletters have been utilized. TV presence, articles in local and national newspapers as well as in the European Parliament magazine, and dissemination workshops organized at the end of the project have completed our array of dissemination activities.
The foreground developed by EuRhythDia will be exploited scientifically; numerous scientific publications are currently under preparation in addition to those that have already been published, in our effort to disseminate our scientific results. A list of publications – already published as well as pending and in preparation – has been provided with this final report.

Exploitation of EuRhythDia results will also be exerted through intellectual property asservation; one application for a patent is currently being evaluated by the institution concerned for submission to the European Patent Office.

Additionally, one partner has implemented methods for the analysis of periodicity and compliant management of clinical data, which will be available as statistical software package for future use.

The compound screening performed within work package 5 revealed novel compounds modulating target enzymes and receptors defined by the consortium. These compounds will be available for scientific and commercial exploitation through the manufacture of basic pharmaceutical products and pharmaceutical preparations.

Exploitation also relates to the presentation of information on diabetes risk and its control through lifestyle intervention in night shift workers. EuRhythDia researchers hope that this will result in more alertness for the problem of insulin resistance in shift workers, as well as better and more specific recommendations for preventive measures.

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