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RESEARCH ARTICLE

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Metabolomic patterns associated to QTc interval in shiftworkers: an explorative analysis

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ABSTRACT

Objectives: ^{1}H NMR-metabolomic approach was used to investigate QTc interval correlation with plasma metabolic profiles in shiftworkers.
Methods: Socio-demographic data, electrocardiographic QTc interval and plasma metabolic profiles from 32 male shiftworkers, were correlated by multivariate regression analysis.
Results: We found a positive correlation between QTc interval values, body mass index, glycemia and lactate level and a negative correlation between QTc interval and both pyroglutamate and 3-hydroxybutyrate plasma level.
Conclusions: Our analysis provides evidence of the association between clinical, metabolic profiles and QTc interval values. This could be used to identify markers of early effects and/or susceptibility in shiftworkers.

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KEYWORDS
Metabolomics; QT prolongation; shiftwork

Introduction

In the electrocardiographic record, the QT interval is the interval between the onset of the QRS complex that is the earliest indication of ventricular depolarization, and the end of the T wave, that is the latest indication of ventricular repolarization (Rautaharju et al., 2009). Prolongation of the heart rate adjusted QT interval (LQTc) can predict a polymorphic ventricular tachycardia known as "*Torsades de pointes*" that consists in the oscillation of the QRS complex around the isoelectric line, and is associated with syncopal episodes, and possible evolution to ventricular fibrillation and sudden death (Giorgi et al., 2010).

LQTc can be either congenital or acquired. Congenital forms are inherited as Mendelian characters, and are associated with an elevated risk of arrhythmogenic sudden death (Kannankeril et al., 2010). Drugs that can interfere with ionic flow through the K + channels, such as antipsychotics, are by far the most common cause of acquired LQTc, which is in turn associated with a significantly increased risk of malignant ventricular arrhythmia (Isbister & Page, 2012). Some authors speculated that the QTc interval can be influenced by shiftwork, and particularly by working unpredictable and nonstandard working hours, and proposed monitoring QTc as an early marker of cardiovascular disorders (CVD) among shiftworkers (Meloni et al., 2013; Murata et al., 1999). However, LQTc can be also considered as a marker of susceptibility in workers exposed to different working schedules. Considering the global burden of CVD and its impact in morbidity, mortality and health costs (WHO, 2011), and the relevant proportion of shiftworkers in industrialized countries (16–20%), introducing workplace screening of early adverse cardiovascular effects and cardiovascular hypersusceptibility can be of special interest in modern occupational health, particularly if coupled with the use of metabolomics and other OMICS technologies (Rasmiena et al., 2013; Vlaanderen et al., 2010). In particular, metabolomics, as "a global holistic overview of the personal metabolic status", can be useful to identify individuals at risk due to the interaction between genetic, environmental, occupational and lifestyle factors (Holmes et al., 2008; Lindon & Nicholson, 2008), by means of the quali-quantitative analysis of a large number of metabolites that are stable compounds with a unique structure and a biological role (Dunn et al., 2011; Wishart et al., 2009).

To the best of our knowledge, only one report has been published which applied a metabolomic approach to assess metabolic patterns associated with an increase in risk of developing QTc prolongation in Guinea pigs exposed to increasing doses of sparfloxacin, a compound belonging to the third-generation family of Fluoroquinolones, known to induce QTc prolongation (Park et al., 2013). No metabolomics studies have been published so far to identify LQTc related metabolic factors in humans.

The aim of the present study was to assess whether QTc interval values correlate with the metabolic profile in a working population with unpredictable and nonstandard working hours, using a ^{1}H nuclear magnetic resonance (NMR)-metabolomics approach.

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Methods

Study population

During the 2013 annual workplace health surveillance program, we examined 32 male workers of a cargo-handling trans-shipping company. All subjects agreed to participate in the study and gave written informed consent prior to participation. For each study subject, we recorded demographic and lifestyle variables, such as alcohol intake and smoking habits, health history and clinical variables, including body mass index (BMI), systolic and diastolic pressure, glycemia, heart rate and the electrocardiographic QTc interval.

Electrocardiograms (ECG) were performed between 07:00 and 10:00 a.m. using a P80 Power-ESAOTE machine (Florence, Italy) the first day upon return to work or after no more than one day shift. Study subjects had to rest in the supine position for 10 min. As QT interval is influenced by heart rate, for each study subject, RR intervals on the ECG were measured in real time for heart rate correction. The QTc interval was automatically calculated by the equipment from the RR and QT intervals on the ECG according to the Bazett's square root formula ($QTc = QT/\sqrt{RR}$), in V1–V6, from the onset of QRS complex to the end of the T wave at the point in which it returns to the isoelectric line, and visually validated according to the recommendations from the American Heart Association, Electrocardiography and Arrhythmias Committee, Council on Clinical Cardiology, the American College of Cardiology Foundation and the Heart Rhythm Society (Rautaharju et al., 2009). We considered a QTc length less than 430 ms as normal, between 430 and 450 ms as borderline, and greater than 450 ms as prolonged, according to published reference values (Yap & Camm, 2003).

Metabolomics approach

Our metabolomics approach consists of two sequential phases: (1) the use of high resolution ^{1}H NMR spectroscopy to profile the full set of metabolites in plasma samples and (2) the interpretation of spectral data, together with the clinical data, by principal component analysis (PCA) and partial least squares (PLS) regression (Barker & Rayens, 2003). Results were visualized in the score 2D-plot, where samples are projected in the space spanned by the principal components (PCs) that summarized all the variables giving higher importance (weight) to those exhibiting higher covariance, and the corresponding loading (weight) plot where the variables are reported. Loading plot can be depicted as scatter 2D-plot or as column plot that shows loading correlation values along one component.

Blood sample collection

Blood samples were collected in heparin vials before executing the ECG, and immediately centrifuged at 4000 rpm for 15 minutes; the supernatant was then divided into 800 µl plasma aliquots and stored at −80 °C.

Plasma chloroform/methanol/water extraction

All plasma samples were thawed and centrifuged at 12,000 rpm for 10 min; the supernatants were processed using chloroform/methanol/water extraction. Plasma aliquots were added with 2.4 ml of chloroform/methanol (1:1 vol/vol) and 350 µl of H_2O, vortexed for 30 s, and centrifuged at 4500 rpm for 30 min. After centrifugation, the hydrophilic fractions were collected. Approximately 1 ml of the hydrophilic fraction was dried overnight using a Speed Vacuum Concentrator (Eppendorf) and stored at −80 °C until NMR analysis.

^{1}H NMR spectroscopy

^{1}H NMR experiments were performed using a Varian UNITY INOVA 500 spectrometer operating at 499.839 MHz for proton, equipped with a 5-mm double resonance probe (Agilent Technologies, Santa Clara, CA). At the moment of analysis, dried hydrophilic plasma extracts were re-dissolved with 650 µl of D_2O (99.8%, Cambridge Isotope Laboratories Inc., Andover, MA) containing the internal standard sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP. 98 atom % D, Sigma-Aldrich, Milan, Italy) at a 0.3-mM final concentration, and transferred into a 5-mm NMR tubes. ^{1}H NMR spectra were acquired at 300 K with a spectral width of 6000 Hz, a 90° pulse, an acquisition time of 2 s, a relaxation delay of 2 s and 256 scans. The residual water signal was suppressed by applying a presaturation technique with low power radiofrequency irradiation for 2 s.

^{1}H NMR data processing and multivariate statistical analysis

^{1}H NMR spectra were imported in the MestReNova software (Version 7.1.2. Mestrelab Research S.L., Santiago de Compostela, Spain), preprocessed with line broadening of 1 Hz, zero-filled to 64 K, and Fourier transformed. All spectra were phased and baseline corrected. Chemical shifts were referred to the TSP single resonance at 0.00 ppm. Assignment of NMR resonances to metabolites was based mainly on literature data (Wishart et al., 2009), and on the database of Chenomx NMR suite 7.1 (Chenomx Inc., Edmonton, AL, Canada). To make information contained in the ^{1}H NMR spectra suitable to perform statistical analysis, they were reduced into consecutive integrated spectral regions (bins) of equal width (0.04 ppm) corresponding to the region 0.50–8.62 ppm. The spectral region between 4.26 and 6.22 ppm was excluded from the analysis, because it presents artifacts from water signal suppression. The integrated area within each bin was normalized to a constant sum of 100 for each spectrum, in order to minimize the effects of variable concentration among different samples. In this manner, 150 variables that represent the spectral profile for each sample were obtained. Furthermore, data from clinical reports (age, BMI, systolic and diastolic blood pressure, glycemia) were added as continuous variables to the NMR data. To distill the latent information contained in the spectral and clinical data related to QTc interval, we performed multivariate statistical analysis with the aid of SIMCA-P + program (Version 13.0. Umetrics, Malmö, Sweden). Mean-centering was applied column-wise, spectral data were Pareto scaled, while clinical data were scaled to unit variance. PCA and PLS regression modeling and its

orthogonal (OPLS) implementation (Eriksson et al., 2013) were performed. As QTc value was the unique outcome, the PLS-1 algorithm was used. Model quality was evaluated on the basis of the R^2 (goodness of fit) and Q^2 (goodness of prediction determined through the default leave-1/7th-out cross validation) parameters.

Ethics statement

The study protocol was notified to the competent Institutional Review Board, the Ethics Committee of the Cagliari University Hospital. Due to the observational nature of the study, in absence of any additional invasive procedure beyond the routine mandatory health screening protocol, and in absence of any involvement of therapeutic medication, based on the guidelines of the Italian Drug Agency (2008) no formal approval was required. Nonetheless, all participating subjects gave written informed consent prior to participation.

Results

Selected characteristics of our study population are reported in Table 1. Six study subjects (14%) had a condition potentially associated with a prolonged QTc interval (four hypertension, one ischemia, one diabetes); none reported undergoing medication with drugs potentially affecting the QTc interval. QTc intervals in the study population were within the normal range in 24 subjects, borderline in 6, and prolonged in 2.

Table 1. Clinical data of the study population.

	Mean	SD[a]	Min	Max
Age (years)	44	6.8	32	60
Height (cm)	173	5.4	162	183
Weight (kg)	76	9.5	53	98
BMI (kg/m^2)	26	3.1	18	31
QTc (ms)	414	22.5	369	453
Systolic blood pressure (mmHg)	122	9.1	110	150
Diastolic blood pressure (mmHg)	80	6.2	70	90
Heart rate (f/m)	66	9.6	52	90
Glycemia (mg/dl)	91	11.9	73	130

[a]Standard deviation.

Plasma hydrophilic extracts were analyzed by ^{1}H NMR spectroscopy. Figure 1 shows the aliphatic region of a representative ^{1}H NMR spectrum including assignment of the major metabolites identified.

To investigate sample characteristics a PCA of spectral and clinical data was performed, the first two PCs accounted for 47% of the variance. As shown in Figure 2(A), all samples lie within the confidence bounds indicating the absence of outliers. We did not observe clusters or trends associated with QTc. The descriptive quality of the PCA model was assessed by the good relationships between metabolites and clinical data that can be evinced by the visual analysis of the loading plot in Figure 2(B). Along PC1 (*x*-axis), glycemia and glucose are close each other and opposite to metabolites involved in energetic supplies such as BCAA (branched chain amino acids); in this latter multivariate region of the plot, 3-hydroxybutyrate, lysine and pyroglutamate are also present. BMI, age, systolic and diastolic blood pressure form a cluster with lactate along the second component (*y*-axis).

In the attempt to correlate QTc interval values with ^{1}H NMR spectral profiles, with the aid of clinical data, we performed an OPLS with QTc values as the outcome. Figure 3(A) shows the OPLS score plot, where a pattern of QTc values can be observed along the predictive component (*x*-axis). Results indicate that BMI, lactate, age, glycemia, glucose and systolic blood pressure are positively correlated to QTc values, while lysine, pyroglutamate, 3-hydroxybutyrate and acetate are inversely correlated (Figure 3B). OPLS metabolites and clinical data exhibiting the strongest (positive or negative)

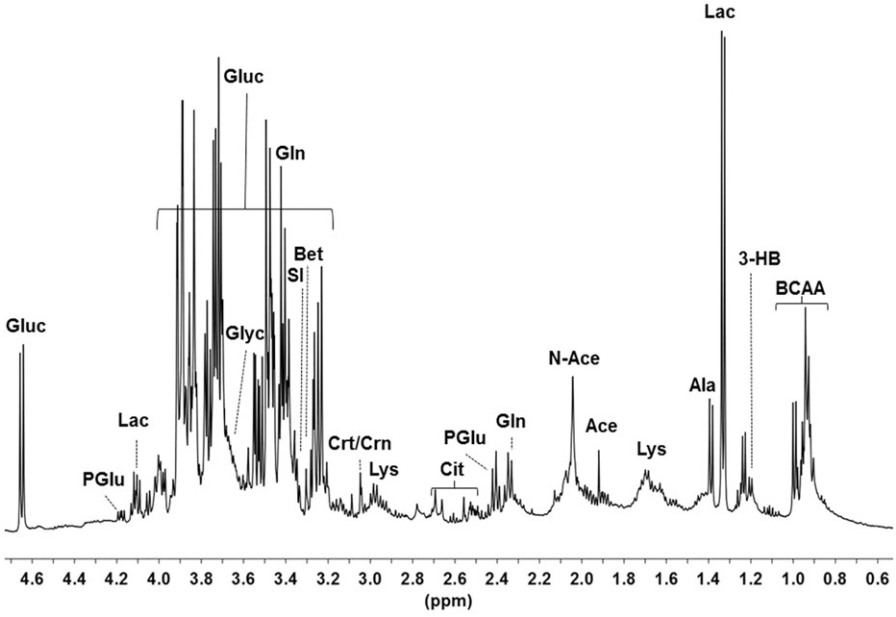

Figure 1. Aliphatic region of a representative ^{1}H NMR spectrum of a plasma hydrophilic extract in D_2O. Major assignments are reported. Branched chain aminoacids (BCAA: isoleucine, leucine and valine), 3-hydroxybutyrate (3-HB), lactate (Lac), alanine (Ala), lysine (Lys), acetate (Ace), N-acetyl groups (N-Ace), glutamine (Gln), pyroglutamate (PGlu), citrate (Cit), creatine/creatinine (Crt/Crn), betaine (Bet), *scyllo*-inositol (SI), glucose (Gluc) and glycerol (Glyc).

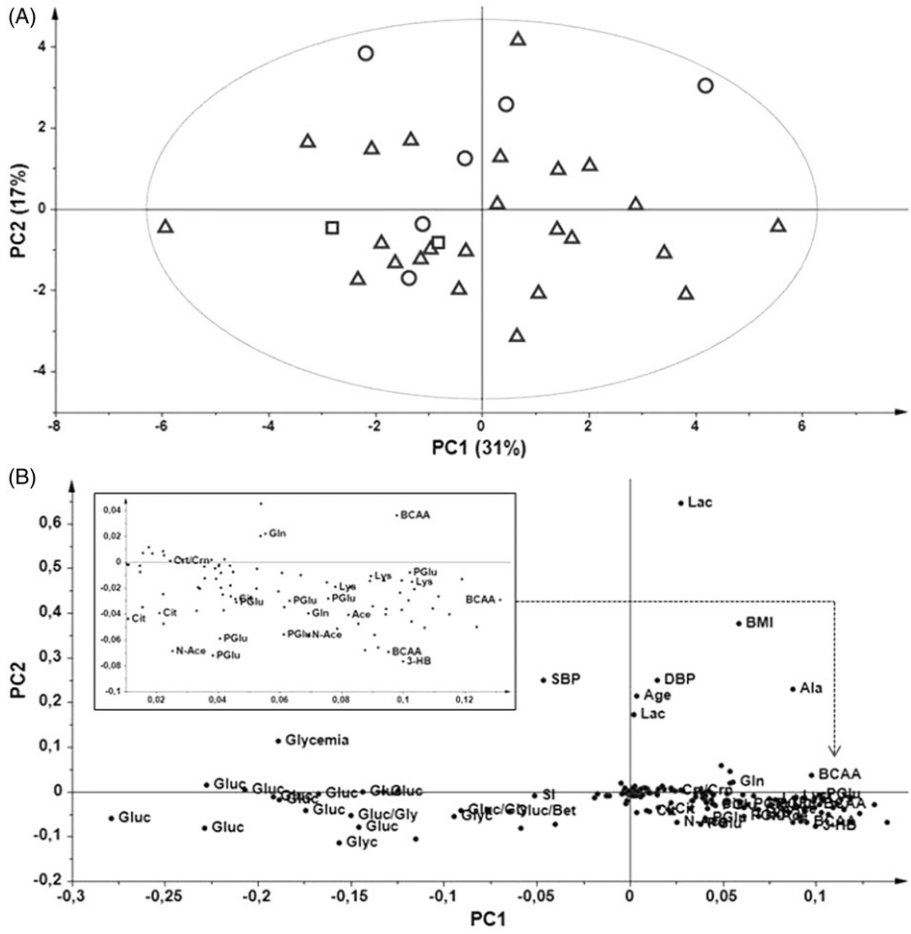

Figure 2. PCA model of subjects with different QTc interval values. (A) Score plot of samples (boxes: subjects with prolonged QTc interval values, circles: borderline, and triangles: normal). (B) Loading plot of variables (spectral and clinical data) with enlarged region. Abbreviations: Branched chain aminoacids (BCAA: isoleucine, leucine, and valine), 3-hydroxybutyrate (3-HB), lactate (Lac), alanine (Ala), lysine (Lys), acetate (Ace), N-acetyl groups (N-Ace), glutamine (Gln), pyroglutamate (PGlu), citrate (Cit), creatine/creatinine (Crt/Crn), betaine (Bet), *scyllo*-inositol (SI), glucose (Gluc), glycerol (Glyc).

correlation with QTc interval values are reported in Table 2. The combined use of spectral data and clinical information allowed a good model quality ($R^2Y = 0.90$, $Q^2 = 0.69$) to be achieved.

Discussion

In this study we found, using a ^{1}H NMR-based metabolomic approach to characterize biochemical patterns related to QTc interval in shiftworkers, a positive correlation between QTc interval values and several clinical and demographical data (such as BMI, glycemia, systolic blood pressure and age) together with some metabolic ones (such as lactate and glucose metabolites). Our findings are consistent with previous reports in underlining a putative association of QTc prolongation with age, obesity, metabolic syndrome, diabetes and also with hyperglycemia and insulin resistance (Faramawi et al., 2008; Li et al., 2009; Queen et al., 2012). A correlation between blood glucose concentration and QTc interval has also been confirmed in non diabetic patients independently on the aforementioned cardiovascular risk factors, such as dyslipidemia and obesity (Grandinetti et al., 2005; Kubiak et al., 2010). Long QTc in subjects affected by hypertriglyceridemia, hyperglycemia and obesity, might be in fact related to an increase in oxygen consumption and in heart rate in these subjects (el-Gamal et al., 1995). An association between systolic blood pressure and QTc interval has also been reported, suggesting a possible role of hypertension in LQTc mediated by ventricular arrhythmias (Barison et al., 2011). In the present study, systolic blood pressure values, but not diastolic ones, were positively associated with LQTc.

Our results also indicate that QTc interval values are inversely correlated in our shiftworkers cohort to pyroglutamate, 3-hydroxybutyrate and acetate.

Pyroglutamic acid can be found in several food items such as cheese, fruit, meat and vegetables, having been suggested by animal and *in vitro* studies a protective role against neuropathy (Silva et al., 2001). Among its several functions, some authors recently suggested a possible anti-diabetic action of pyroglutamic acid, in an animal setting, related to its ability to improve glucose tolerance either by down-regulating glycolysis and gluconeogenesis, or by suppressing the increase in plasma glucose levels (Yoshinari & Igarashi, 2011).

3-Hydroxybutyric acid is a ketone body resulting from the conversion of cystathionine to cysteine in the methionine-to-glutathione pathway. Like all the other ketone bodies (acetoacetate and acetone), its levels in blood and urine increase in ketosis and may be used in diabetic patients as a marker of ketoacidosis.

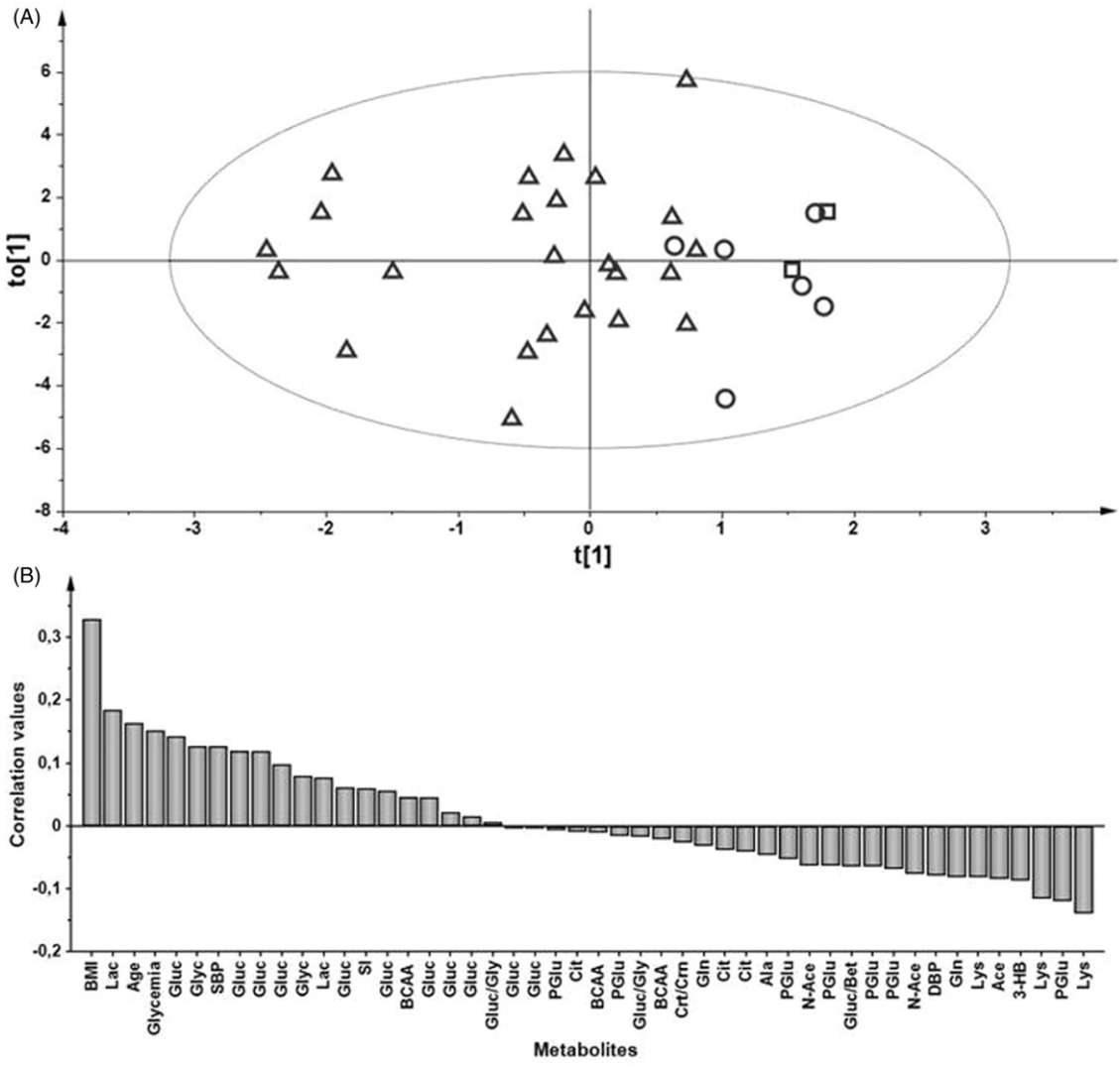

Figure 3. OPLS model with QTc interval values as response. (A) Score plot of samples (boxes: subjects with prolonged QTc interval values, circles: borderline and triangles: normal). (B) Loading column plot of variables (spectral and clinical data) along the predictive component t[1].

Table 2. OPLS metabolites and clinical data correlated to QTc interval values.

Directly correlated	Inversely correlated
BMI	Lysine
Lactate	Pyroglutamate
Age	3-Hydroxybutyrate
Glycemia	Acetate
Glycerol	Glutamine
SBP	
Glucose	

Acetate is implicated in fatty acid synthesis and in lipogenesis in the adipose tissue. It has been recently proposed as a mediator in reducing appetite via a central homeostatic mechanism (Anastasovska et al., 2012; Frost et al., 2013). Glucose and acetate plasmatic concentrations seem to be associated, even if a causative relationship has not been so far proposed. Their biological link may be an enzymatic one and it may rely on the functional insulin mediated-regulation of acetylcoenzyme A synthetase (ACAS; involved in the conversion of acetate to acetyl-CoA), existing some evidence of a conversion between acetate and glucose via acetyl-CoA (Akanji et al., 1989).

The overall metabolic pattern here described seems to be strictly interrelated with glycemic homeostasis, suggesting a causative relationship between elevation of blood glucose level, QTc prolongation and shiftwork.

The described correlation among QTc prolongation and higher values of systolic blood pressure, BMI, blood glucose (expressed either as glycemia and NMR signals, independently from absence/presence of insulin resistance), together with the inverse relation to pyroglutamate, 3-hydroxybutyrate and acetate, underlines a common metabolic way that could impair autonomic activity influencing QTc interval, even without neuropathy (Fiorentini et al., 2010). Moreover, several studies, based on intensive glycemic control, suggest a possible causative correlation between a hypo/hyper-glycemic status and an excess of cardiovascular morbidity and mortality in patients with type 1 and 2 diabetes via arrhythmias and modification of cardiac autonomic activity (The ACCORD Study Group, 2011).

There is a growing need to understand the molecular basis of CVD in order to identify effective screening tools to monitor cardiovascular risk factors at the general population and occupational health level. CVD is, in fact, the major contributor to global mortality and morbidity in industrialized countries, and the related economic and healthcare costs

represent a major public health policy concern (WHO, 2011). It is well known that cardiovascular risk is strongly related to obesity, elevated blood pressure, hyperglycemia and dyslipidemia. Early asymptomatic factors, such as LQTc, are less known. QTc is, in fact, considered as a prognostic factor of arrhythmia, but the physiopathological mechanisms remain still not fully understood (Park et al., 2013). Shiftwork is reported as a risk factor for the development of CVD (Frost et al., 2009; Murata et al., 1999, 2005). A multifaceted and multivariate approach, linking clinical variables with metabolomic patterns, can represent a useful tool to stratify cardiovascular risk in shiftworkers and identify hypersusceptible subjects together with tailored individual preventive measures to contain the risk.

In researches focused on the understanding of the etiopathological mechanisms of QTc prolongation, metabolomics can in fact allow to acquire qualitative and quantitative information concerning the metabolites that occur under normal circumstances and to detect perturbations in the metabolites as result of environmental exposure (Vlaanderen et al., 2010). Global metabolomic profiling, together with a multivariate statistical approach, may play a key role in discovering metabolic phenotypes that can predict individual QTc prolongation as a consequence of exposure of exogenous risk factors (such as shiftwork, drugs, diabetes, metabolic syndrome and so on). It can make it possible to identify biomarkers of complex and low dose exposure or early effects in human population and detect biochemical changes of disease or exposure prior to more overt signs of adverse health effects (Bonvallot et al., 2013; Dudka et al., 2014; Wang et al., 2012).

As already observed in animal models (Park et al., 2013), this approach might also be useful to identify subjects at risk of developing LQTc also among those under medication, particularly with antipsychotic drugs. Considering the burden of subjects undergoing antipsychotic treatment, public health implications could be relevant. Also, the metabolic genotypes and phenotypes associated to LQTc may be helpful to understanding its origin and to suggest new researches to identify patterns of susceptibility in shiftworkers.

Some limitations need to be considered interpreting our findings. The sample size of our study population and the observed prevalence of LQTc are too small to allow any general inference to be drawn. Moreover, we did not have a reference group to test differences in metabolic profiles compared to shiftworkers, and we did not conduct a longitudinal analysis to study modifications of QTc in relation to metabolic and clinical phenotypes. In spite of such limitations, our explorative analysis provides initial evidence for the association of clinical and metabolic profiles with the QTc interval which could be useful in further studies to identify markers of early effects and/or susceptibility, especially in shiftworkers.

We will address further efforts in this direction. Our findings support the application of a metabolomics approach to the study of exogenous risk factors of QTc prolongation in humans. This is the first metabolomic study, at the best of our knowledge, conducted in humans in relation to QTc interval and it can represent a first step in future research field in both occupational health as well as in cardiology and pharmacology.

Disclosure statement

None of the authors declare any competing interests in the matters related to this paper.

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