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Influence of shift work on the urine metabolite profile in female nurses

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on the urine metabolite profile in female nurses

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List of Abbrevations

Abbreviation	Meaning	Page
BCAA	Branched chain amino acids	14
CLOCK/BMAL1	Circadian Locomoter Output Cycles Kaput/Brain and Muscle Aryl hydrocarbon receptor nuclear translocator-Like 1	9
CV	Coefficient of variance	12
DGUV	Deutsche Gesetzliche Unfallversicherung	15
EHHADH	Enoyl-CoA Hydratase and 3-Hydroxyacyl CoA Dehydrogenase	9
FAO	Fatty acid oxidation	14
FIA-ESI-MS/MS	Flow injection analysis-electrospray ionization-tandem mass spectrometry	12
IARC	International Agency for Research on Cancer	7
IDQ	Identification and Quantification	12
LOD	Limit of detection	12
MCTQ	Munich Chronotype Questionnaire	9
$MCTQ_{MS}$	Local time of mid-sleep assessed by the Munich Chronotype Questionnaire	9
$MCTQ^{Shift}$	Munich Chronotype Questionnaire for shift workers	9
MSF_{sc}	Local time of mid-sleep on free days corrected for sleep dept accumulated over the work week	9
NAD	Nicotinamide adenine dinucleotide	9
NAMPT	Nicotinamide phosphoribosyl transferase	9
RBN	Regression based normalization	11
SCN	Suprachiasmatic nuclei	8
SOP	Standard operating procedures	11

Summary (German)

Im Zuge dieser Arbeit soll der Einfluss von Schichtarbeit, unter Berücksichtigung des jeweiligen Chronoyp, erhoben werden. Die Ergebnisse dieser Forschung soll zum zur Verbesserung der Gesundheit und Arbeitsbedingungen von Schichtarbeitern dienen.

Verschiedene Studien konnten bisher die negativen Auswirkungen von Schichtarbeit auf die Gesundheit zeigen. Obwohl einige dieser Studien zum Teil den Einfluss auf den Metabolismus und die beeinflussten Stoffwechselwege zeigen, wurde bisher nicht der individuelle Chronotyp in diesem Zusammenhang berücksichtigt. Zusätzlich war die verfügbare Information über den Einfluss von Lagerungsbedingungen aus das Metabolitprofil in Urin eher spärlich.

Das übergeordnete Ziel dieser Arbeit war die Erforschung des Einflusses von Schichtarbeit auf den Metabolismus von Krankenschwestern unter Berücksichtigung des jeweiligen Chronotyp. Durch Auswertung der gesammelten Daten sollen die Grundlagen geschaffen werden, die täglichen Abläufe sowie die Gesundheit von Schichtarbeitern weiter zu verbessern. Des weiteren wurde untersucht, in wie fern die Lagerungsdedingungen Einfluss auf die Metabolitenkonzentrationen der Proben hatte, welche während der Schichtarbeit gesammelt wurden.

Um potentielle Einflüsse von Nachtarbeit aufzuzeigen, wurden Metabolit-Konzentrationen in Urin Proben aus der Tag- und Nachtschicht verglichen. Dazu wurden vor allem Messergebnisse von den ersten Proben nach dem Aufstehen verwendet. Separate Analysen für frühe-, mittlere- und späte Chronotypen wurden durchgeführt. Verschiedene Normalisierungsmethoden wurden verwendet um unterschiedliche Urinkonzentrationen zu berücksichtigen. Um die Einwirkung von Lagerungsbedingungen auf die Metabolitkonzentrationen zu untersuchen, wurden Urinproben von verschiedenen Spender vereinigt und verschiedenen Umwelteinflüssen wie Temperatur und Zeit ausgesetzt. Die darauf hin gemessenen Werte wurden mit Werten von umgehend eingefrorenen Proben verglichen.

Bei einem Vergleich der Tag- und Nachtschicht konnten, abhängig vom jeweiligen Chronotyp signifikante Unterschiede bezüglich der Metabolitkonzentration festgestellt werden. Innerhalb der Gruppe der frühen Chronotypen waren am meisten Änderungen innerhalb der untersuchten Metabolitkonzentrationen zu beobachten. Dabei waren vor allem mittlere- und langkettige Acylcarnitine wärend der Nachtschicht betroffen. Dies legt eine beeinträchtigte Fettsäureoxidation durch Schlafmangel nahe. Die Konzentrationen von ungefähr 80% der Acylcarnitine, Fette und Aminosäuren waren unter den simulierten Lagerbedingungen stabil.

Im Zuge dieser Forschungen konnten wir feststellen, dass der jeweilige Chronotyp in der Tat einen Einfluss darauf hat, wie der Stoffwechsel auf Schichtarbeit reagieren. Bestimmte Klassen an Stoffwechselprodukten sind hier mehr betroffen zu sein als andere. Dabei scheint die Lagerung in Kühltaschen wärend und nach der Probennahme nur einen untergeordneten Einfluss auf die Metabolitkonzentration gehabt zu haben.

Ein potentielles Einsatzgebiet der gewonnenen Erkenntnisse könnte die Einbeziehung des Chronotyp bei der Planung der Arbeitsschichten sein. Des Weiteren können die identifizieren, durch Nachtarbeit am stärksten betroffenen Metabolite als Vorauswahl für tiefgreifendere Forschung verwendet werden.

Executive Summary

In the course of this work, we assessed the impact of night shift work with respect to the individual chronotype. This research can then be utilized to improve the health and professional life of shift workers.

Various studies showed the detrimental impact of shift work on health. Although some of these studies investigate the metabolic impact and affected pathways, little is known on the effects of shift work considering the individual chronotype. Information on targeted metabolite profiles in urine in this regard was sparse. Also, information on the impact of urine storage conditions on the metabolite profile when using the targeted technology, was lacking.

The primary goal of this study was to assess the metabolic impact of night shift work of female nurses considering the individual chronotype. By analyzing our collected data, we aim to improve the daily life and health of shift workers. Furthermore, the impact of urine storage conditions on the metabolic profile was investigated to appraise the extend to which storage might have affected the collected urine samples during shift working periods.

To uncover potential metabolic impacts of working at night, the urine metabolite profiles during night shift and day shift in shift working nurses were compared. Here, the measurements based on urine samples donated after waking up were used. The analysis was furthermore stratified by individual chronotype into early, intermediate and late chronotype. Different normalization approaches where employed to take urine concentration into account. To assess the influence of sample storage, pooled urine was kept under various urine storage conditions. Metabolite concentrations were then compared to samples immediately which were immediately frozen.

Depending on the individual chronotype, differences in metabolite levels could be observed when comparing dayshift to night shift metabolite levels. Individuals in the early chronotype stratum were affected the most. Medium- and long -chain acylcarnitines in the early chronotypes appeared to be affected most during night shifts. This suggests an impaired fatty acid oxidation due to sleep restriction. About 80% of acylcarnitines, lipids and amino acids were stable at the examined storage conditions

In the course of this study we found that the individual chronotype does influence the way the metabolism reacts on shift work. Certain classes of metabolites appear to be more affected then others. Urine storage on cool packs appeared to have in our case a negligible influence on the above identified metabolites.

A potential application method could be to consider the personal chronotype when planning shift schedules. The identified metabolites could be furthermore used as a preselection for more in depth research.

Preface

Sufficient sleep is crucial for human wellbeing (1). During sleep, the body does not only conserve energy due to lower oxygen consumption but also restores biosynthetic processes and allows repair and repletion of various cellular components, tissues and organs including the brain (2–4).

Continuous sleep deprivation and impaired sleep quality, frequent side effects of night shift work and rotating shifts, are therefore detrimental for human health and can lead to increased risk of various metabolic diseases including diabetes (5,6). An expert panel of the International Agency for Research on Cancer (IARC) concluded that circadian disruption in female shift workers and flight attendants could also be carcinogenic (7).

Reasons for the higher occurrence of adverse health issues in shift workers have not yet been elucidated. Impact of the chronotype, i.e. a person's natural inclination to be most alert at a certain time of day, and preferred sleep times on metabolism and ultimately health itself is also unclear. Research in female shift workers revealed that early chronotypes, i.e. persons with morning preference, working in night shifts have a significantly higher risk of breast cancer than late chronotypes (8). Two studies assessed the effects of sleep deprivation on human blood metabolome (5,9). While they have advanced the metabolic understanding of night shift work, the strictly regulated laboratory environments of these studies might have not necessarily reflected the daily work-life routine. This urges for a research in real life settings using easy-to-collect biological samples such as urine that will take into account personal lifestyle, eating habits and preferred timings of sleep of the study participants (1).

Large-scale of targeted metabolite profiling of human blood samples has been applied for the identification of biomarkers that are characteristic of a disease or lifestyle (10–13). However, further research is required to identify biomarkers and respective metabolic pathways affected by shift work with respect to individual chronotype, especially using urine samples in a real-life study. This would provide the means for early health prevention like the application of personalized work schedules according to the individual chronotype and lessen the negative influence of shift work on health (14).

The first aspect of this work was to assess the impact of storage conditions (e.g. cool pack for up to 24 hours) on the stability and quality of urine metabolite concentration profile (15). The intention was to evaluate to what extend storage of urine sample might influence measurements of metabolite levels. In the second study, all 3640 spontaneous urine samples collected from 100 nurses were kept for maximal 24 hours on cool packs and immediately stored afterwards at -80°C (16). Subsequent measurements were undertaken to assess metabolite profiles in the donated urine. The insights from the first study helped to gauge to what extend the observed metabolite changes during night shift might be due to potential storage influences.

With this work we aim to provide metabolic impacts of night shift work with respect to individual chronotype. Insights presented here will help to improve the health and daily life of shift workers.

1 Introductory Summary

1.1 Shift work

Even short periods of night shift reduce sleep quality, which in the long run can give rise to severe health issues (6,17,18). Since the introduction of artificial lighting in the daily life, work around the clock has become a common phenomenon. In 2016, 17.4% of the employees in Germany were shift workers, meaning they worked outside of the "normal" working hours from 8 am to 6 pm on weekdays. This included 15.6% of female and 19.1% of male working population (19). In Europe, this corresponded to 18.6% of employees, of which 17.4% were females and 19.8% males (19).

Shift work is especially prominent in health care, where patients are dependent on around-the-clock intensive care provided by health professionals (20). Of the about 5.4 million people working in health care and social services sectors in 2018, 4.2 million were women (21). In comparison to day shift nurses, night shift nurses have more difficulties to adapt to their respective shift schedules (22). Additionally, younger persons tend to be more frequently enrolled in shift work and the tolerance towards shift work further decreases with age (16,23). Working during physiologically unusual hours can lead to a number of detrimental side effects including abnormal eating pattern and increased exposure to artificial light that subsequently affects circadian rhythm (24,25).

1.2 Circadian Rhythmicity

1.2.1 Circadian regulation

Regular exposure to environmental light is crucial for the synchronization of sleep-wake cycles, also known as circadian rhythmicity (26,27). The master circadian clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus plays the central role in the regulation of daily rhythms of sleep-wake cycles (28–30). The SCN consists of neurons that generate cell-autonomous circadian oscillations in gene expression and neural activity (14). Their activity is synchronized by environmental factors such as light and dark cycles (31). Light signals are transmitted to the SCN and subsequently converted to chemical signals which in turn impact the phase of the clock gene expression in peripheral cells (31). Temperature, sleep and wake timing, as well as the energy metabolism are regulated by the SCN (14,30,32).

The first clock gene developed in cyanobacteria, approximately four billion years ago (33). It served to protect the organism from dangerous ultra violet rays but also allow their survival through photosynthesis (34). The elucidation of the molecular mechanisms controlling the circadian rhythm earned J. Hall, M. Rosbash and M. Young the Nobel Prize in Physiology or Medicine in 2017. The expression of clock genes follows a self-sustained, near 24 hours-long rhythm that is synchronized by internal and external time cues. This molecular clock acts through complex translational and transcriptional feedback loops comprising positive and negative elements (35).

One of the specific transcriptional feedback loops includes the gene encoding nicotinamide phosphoribosyl transferase (NAMPT) (16). Its circadian expression is regulated by the transcription factor complex Circadian Locomoter Output Cycles Kaput/Brain and Muscle Aryl hydrocarbon receptor nuclear translocator-Like 1 (CLOCK/BMAL1) (36,37). NAMPT is an integral part of the fatty acid oxidation and a rate limiting step in the Nicotinamide adenine dinucleotide (NAD+) salvage pathway (38). As a critical coenzyme of hundreds of metabolic enzymes, the intracellular NAD pool provides hydrogen atoms for oxidation-reduction reactions, production and maintenance of energy stores, and protects from metabolic diseases and aging (37). Another circadian enzyme involved in fatty acid metabolism is the bifunctional enzyme Enoyl-CoA, Hydratase/3-Hydroxyacyl CoA Dehydrogenase (EHHADH) that metabolizes medium chain fatty acids that are transported to mitochondria through carrier protein carnitines (39). The expression of EHHADH in mice is regulated by the circadian clock gene Bmal1 (40), suggesting that lipid degradation could be altered when the exposure to natural light is not followed.

1.2.2 Chronotype

The circadian regulation occurs on an intrinsic, molecular level. Although factors like the personal preference concerning sleep and wake cycles take place on a more individual level, the potential connection between molecular and personal conditions needs to be considered. The individual preference for the sleep-wake cycles is defined as chronotype. It is regulated by the circadian clock, a temporal program inherent to most organisms , that takes place both on the level of gene expression and the behavioral level (41,42). Circadian clock is not necessarily restricted to stimulation from external zeitgebers (daily environmental signals) such as light exposure and food but also the genetic variations in the clock genes (42). All these factors affect the personal classification into a certain chronotype category, which may range from *extreme early* to *extreme late* chronotype (41). The personal chronotype influences to which the extent shift work could affect the health and potential detrimental health outcomes of an individual (8).

To assess the chronotype of shift workers, an adaptation of the Munich Chronotype Questionnaire (MCTQ), the Munich chronotype questionnaire for shift-workers ($MCTQ^{Shift}$), is employed (43). Similar to MCTQ, the chronotype is assessed from sleep behaviour on the local time of mid-sleep on free days and further corrected for sleep debt accumulated over the work week (MSF_{SC}) to take into account a potential sleep depth which accumulates during working days (43,44). In contrast to the MCTQ, which assumes standard working hours, the $MCTQ^{Shift}$ considers each shift period (6). The mid-sleep time point is defined as the time of the day when the half of the sleep phase has passed (45). For example, a person going to bed at 11 pm and getting up at 7 am would have a mid-sleep time point of 3 am. In our study, the people who cared for small children or used an alarm clock on free days were not considered for participation in order to minimize such external influences on the chronotype (43).

1.2.3 Misalignment

The time of getting up or going to work does not necessarily match work schedules. With the intervention of electric light and sleeping in rooms with light, the “typical” working hours have changed and the sleeping habits need to be adapted (46). Modern working hours have shifted considerably as people became even less constrained by the sun light. The possibility of intercontinental flights and introduction of around-the-clock work have further drifted the biological and social preferences, increased social jet lag (i.e., going to bed and waking up later on weekends) and resulted in a misalignment with healthy circadian clock (41).

Chronodisruption is defined as the desynchronization of the 24-h rhythms resulting in adverse health effects (47). Exogenous and endogenous exposures that are defined as chronodisruptors can disrupt the timing and order, e.g. the organization of physiologic hierarchies (47). Furthermore, the various chronotypes react differently to misalignments and, for example, individuals with earlier chronotypes are more prone to social jet lag (6). While late chronotypes and younger individuals tend to cope better with night shift work, the adaptation and alertness at night shifts decreases in individuals with morning preference and increasing age (23,48,49).

1.2.4 Related health risks

Various aspects of the metabolic syndrome such as diabetes mellitus and cardiovascular diseases have been linked to shift work (50,51). Furthermore, increased risk for breast cancer and obesity have been linked to circadian disruption (52,53).

In addition to these detrimental health outcomes, another obvious consequence of shift work is decreased sleep quality and duration (54). Van den Berg et al and Davies et al found that restricted sleep changes the metabolic profile of acylcarnitines (55,56). Medium- and long-chain saturated acylcarnitines were found to be significantly different when comparing normal sleep to short sleep time periods pointing to possible changes in mitochondrial β–oxidation of fatty acids during sleep deprivation (55). Elevated levels of acylcarnitines in blood were suggested to mediate the effect of short sleep on increased risk of insulin resistance (57,58).

1.3 Urine samples

One of the disadvantages of blood samples in metabolic research is the invasive nature of sample procurement. Urine is easy to obtain, does not necessitate any invasive measures and no special training for retrieval. Various studies employed urine to assess its metabolic changes related to disease status or lifestyle factors (59–61). Urine concentration is, however, highly dependent on factors like water uptake and this can subsequently affect the metabolite concentrations (62). Moreover, the storage conditions of urine influence its metabolite concentrations (63).

1.3.1 Stability of metabolite concentration profiles of urine samples

In urine, the most prominent compounds are water, uric acid, salts and creatinine and may also include bacteria and enzymes (64–66). The metabolic profile of urine is affected by the storage conditions such as temperature or freeze-thaw cycles due to temperature-dependent enzymatic reactions, cell degradation and bacterial metabolism (15,67,68). Previous targeted metabolomics studies in human serum and plasma also showed that metabolite profile is affected by storage conditions. These studies simulated various storage conditions including freeze-thaw cycles, storage on dry ice, wet ice, cool packs or room temperature for 3h, 6h, 12h, 24h or 36h (69,70).

The influence of storage conditions on the urinary metabolite profile has been much less investigated and motivated the work presented in this thesis (15). In laboratory settings, urine handling includes centrifugation and filtration to remove larger cell particles and bacteria, reduction or inhibition of enzymatic processes, and is followed by the storage at temperatures as low as possible (67,71). While these standard operating procedures (SOPs) are common for laboratories, the same procedures are difficult for real life settings since the participants cannot or are not able to adhere to SOPs (16).

1.3.2 Normalization of urine metabolite profiles

A direct interpretation of measured urine metabolite concentrations by a statistical model is not possible since various factors such as urine concentration and the time of donation have to be also considered for a meaningful interpretation. In our study, the total volume of the donated urine samples was not assessed and other approaches were required to assess the urine concentration. Normalization of the metabolite concentration in urine is a common approach to address some of these factors.

One of the most common and easy normalization methods is to relate the metabolite values to creatinine concentration. Here, the measured metabolite concentration is being set relative to the respective creatinine concentration in the same sample (72). Another way to normalize urine metabolite values is to take into account the osmolality of samples, i.e to consider the osmotically active solutes in the analyzed fluid and divide the metabolite concentrations by the respective value (60). A third method is the regression based normalization (RBN) and was used in the *article 2*. In this method, the dilution variation is estimated through a nonlinear regression considering each metabolite's excretion kinetic separately (16,73).

1.4 Metabolomics

1.4.1 Application

A thorough assessment of urinary metabolite concentrations was an important part of two studies presented here. Metabolite degradation was considered when interpreting the metabolic impact of shift work (*article 2*) and urine storage conditions (*article 1*).

The assessment of metabolite concentrations using targeted metabolite analysis in biological tissues like blood and urine is a common technique. The aim was to assess the impact of lifestyle factors like smoking, alcohol consumption or health statuses like pre-diabetes on metabolite profiles and related pathways (10–12). Furthermore, potential metabolic biomarkers to early identify risk groups were investigated (10–12). In the present studies, the targeted technology was used to enable researchers to glean the underlying mechanisms that might cause metabolic changes in urine samples affected by night shift work or storage conditions (15,16).

1.4.2 Technology

Metabolomics is a downstream -omics approach to its more known superordinated terms genomics, transcriptomics and proteomics (74). In contrast to these approaches that analyze genome-wide changes in DNA code, transcript and protein levels, metabolomics aims to assess the metabolites from the biological system under study (75).

Three widely utilized approaches in metabolomics are: 1. Metabolite profiling, which aims to measure metabolites from a predefined class or pathway (76); 2. Metabolic fingerprinting, which is utilized to assess general differences between two biological samples or provide some general information about metabolic regulation (77,78); 3. Metabolite target analysis, the measurement of a predefined group of biochemically annotated metabolites (79,80).

In *article 1* and *2*, metabolite concentrations were measured with the Absolute*IDQ*™ p150 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) using FIA-ESI-MS/MS (flow injection analysis-electrospray ionisation-tandem mass spectrometry) (15,16). The assay focuses on the assessment of acylcarnitines, amino acids, glycerophospholipids, sphingolipids and hexose (81). To account for technical influences, metabolites with a CV higher than 25% or with more than 50% of measurements below the limit of detection, where excluded (15).

1.5 Study design

Since the considerable amount of shift workers are female healthcare providers, the aim of the study population recruitment focused on female clinic personnel of different age that were doing either no shift work (control group) or both day- and night shifts.

To assess the potential influence of storage (room temperature, cool packs, fridge) on the urine metabolite profile, a study was conducted as described in *article 1* (15). Here, the study population consisted of 6 female volunteers, who donated spontaneous urine after a night of fasting. Urine samples were pooled (account for interpersonal differences / no normalization necessary) and stored for 0h, 2h, 8h, and 24h at room temperature (~20°C), 9°C (cool packs), 4°C (fridge) and -20°C

(freezer) respectively and then deep frozen at -80°C. Additionally, up to three freeze (-80°C / 24h) and thaw (room temperature / 2h) cycles were conducted. Each urine sample on the various storage conditions / freeze and thaw cycles was measured four times to account for analytical variation. The same targeted technology as above was used to assess urine metabolite profiles (15).

As described in *article 2*, 100 participants worked either in day shift (25 / day shift block) or both day and night shift (75 / night shift block) with a four week pause in between shifts to account for the menstrual cycle. Throughout that time (up to three consecutive days for day shift block / four consecutive days for night shift block / both at work or at home), participants were asked to collect spontaneous urine and store it in the fridge (~4°C) if possible or cool packs (~9°C). Urine samples were picked up by the study nurse after shift end, but no later than 24 hours after donation and stored at -80°C after processing (aliquoting). Metabolite profiles in urine were measured using Absolute*IDQ*™ p150 Kit (as mentioned above). Additionally, information on sleep timing and diet was collected. Further assessed information included previous or concomitant diseases, medication before and during assessment periods, light quality during shifts and off time and hormone levels at set intervals. Three different types of normalization (creatinine-, osmolality- and regression-based normalization) were performed. Based on the answers of the MCTQ, the personal chronotype was assessed and used for grouping the participants in to early, intermediate and late chronotypes (16).

1.6 Goal of the dissertation

The primary goal of the underlying studies was to find potential biomarkers for a disrupted circadian rhythm and assess the impact of different chronotypes, both of which could contribute to improving nurse health. We compared the differences in the metabolic profile during nightshift and dayshift in shift working female nurses with different chronotype classes. Identified changes in the metabolite concentration might hint to specific metabolic pathways which are more affected by nightshift work depending on the chronotype.

The second part of this thesis assessed the influence of different storage conditions on metabolic profiles in urine. The intention was to consider any potential changes in urine samples collected during the field phase that could be related to different storage conditions. To the best of our knowledge, this was the first study that investigated the impact of storage on urine with targeted metabolomics. Previous studies used non-targeted metabolomics in urine and targeted metabolomics in blood samples (63,69,70,82).

The presented results might be implemented in the day to day working life of shift working personnel. A future implementation of our insights may include potential preventive measures like chronotype-adapted shift patterns. Furthermore, the identified metabolites could be further investigated in subsequent research projects as biomarkers for chronodisruption caused by shift work.

1.7 Content and individual contribution

The contents and presented results of *article 1 and 2* are the integral parts of this dissertation.

In *article 1*, we assessed the impact of various storage conditions on urinary metabolite profile. Pooled urine samples were stored at several temperatures (-80°C, -20°C, 4°C, ~9°C, room temperature), different times (0h, 2h, 8h, 24h) and exposed to freeze-thaw cycles. As previously, the measurements were performed using targeted mass spectrometry. Our results showed that urine samples stored at -20°C and 4°C during 24 hours were equally stable as samples that were frozen at -80°C immediately after collection. A concentration reduction of up to 40% was observed only for arginine, valine and leucine/isoleucine that were stored at 9°C for 24 hours. Moreover, the levels of methionine and hexoses decreased by up to 60% when stored at room temperature for 8h. Overall, these results showed that almost 80% of acylcarnitines, lipids and amino acids are stable at all investigated temperatures and time intervals. Storage conditions thus appear to play only a minor influence on the stability of metabolites and the use of cool packs or even room temperature during 24 hours is satisfactory for downstream analyses (15).

For the project described in *article 1*, the PhD candidate

- Co-designed the study (e.g. deciding on the appropriate urine storage conditions and applying to the ethics committee for study approval);
- Co-coordinated the urine sample logistics and metabolomics measurements and conducted plate design, sample preparation and randomization, pooling of urine / keeping the planned storage conditions, recruiting suitable volunteers;
- Performed the osmolality measurement (e.g. selection of samples, acquiring of measurement devices and actual measurements);
- Conducted all statistical analyses including quality controls;
- Conducted pathway analysis, wrote the first draft of the publication;
- Updated and revised the *article 1*;
- Presented some of these results at numerous national meetings with collaboration partners (“Forschungsbegleitkreis” 2013/2015/2017) and international conference (“Grainau Workshop of Genetic Epidemiology” 2017);

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In *article 2*, we assessed the impact of night shift work on the metabolite profile in 68 female nurses that were classified into an early, intermediate or late chronotype group. First morning urine samples were collected by study participants during up to four consecutive days and analyzed by targeted mass spectrometry. To account for water intake that affects urinary metabolite concentration, metabolite values were normalized using three normalization approaches (creatinine-, osmolality- and regression-based normalization). Using linear mixed effect models we identified 31 metabolites which values were significantly altered between day shift and night shift workers. After chronotype-related stratification and creatinine-based normalization, 11 metabolites were found significantly altered in early chronotypes, none in intermediate chronotypes, and four in late chronotypes. The medium- and long-chain acylcarnitines represented the most commonly affected metabolites in the chronotype-stratified analysis and their levels were increased in early chronotypes working night shifts. This suggested an impaired fatty acid oxidation (FAO) in mitochondria possibly caused by sleep restriction (55). Overall, this study showed a clear effect of night shift work on urinary acylcarnitines and branched chain amino acids (BCAAs) and suggested that the nurses with early chronotypes were the most affected group (16).

For the project described in *article 2*, the author

- Co-designed the study (e.g. deciding on the appropriate urine storage conditions and applying to the ethics committee for study approval);
- Took part in all stages of project planning, execution, analysis and presentation;
- Co-coordinated the urine sample logistics and metabolomics measurement, and conducted plate design, sample preparation and randomization, plausibility checks (visual and statistical / enabling short notice re-measurement) as well as data preprocessing (exclusion of measurements based on technical parameters);
- Performed all statistical analyses and all sample handling (reservation of measurement slots and randomization of samples; quality control including plausibility checks; data analysis, interpretation);
- Wrote first draft of manuscript, updated and revised the paper of *article 2;*
- Presented at international conferences ("Grainau Workshop of Genetic Epidemiology" 2016).

Furthermore, the PhD candidate contributed to extend the DGUV supported project and obtained third party funding that were used for part of the sample measurements and personnel for one year for the main project (*article 2*) and supervised several master and internship students.

2 Discussion

The work presented here has significantly contributed to the field of occupational health. Our results showed that early chronotypes working night shifts are much more sensitive to alterations in the urinary metabolome than other chronotype classes. Moreover, these alterations reflect an impaired catabolism of fatty acids and mitochondrial energy production, which can lead to adiposity and other metabolic diseases including diabetes. Our work has also contributed to the metabolomics field as we found that urine metabolites (majority of amino acids, various phospho- and sphingolipids) are stable under various storage conditions. It showed that the urine is a suitable matrix for field-based study designs in non-laboratory settings.

Shift work, i.e being at work at times of the day that diverge from personal sleep-wake preference, often leads to sleep restriction. Early chronotypes, who would normally already be asleep for hours, need to stay alert to fulfill their duties at night and suffer the most among all chronotypes of sleep deprivation. It has been shown in 2016 that sleep restriction affects metabolism, namely FAO that may lead to insulin resistance (55). We also found that the participants of our study, especially early chronotypes, showed first metabolic signs of impaired FAO in their body due to increased levels of excreted acylcarnitines (16). We could not statistically assess the potential link with early stage of (pre-) diabetes in our study participants due to limited sample size. In addition to altered urinary metabolite levels that are strikingly detectable already after one to four days of night shift work, the changed sleeping pattern, unusual timing of meals and increased exposure to artificial light will intensify the impact of night shift work on the individual health status (54,83,84).

Of all human biofluids, urine is the most easy to collect and would be thus a preferred choice for real life-based study designs where sample collection and storage cannot be performed using the highly regulated environment of a laboratory setting. Filtering of urine samples, subsequent centrifugation and immediate cooling at -80°C, a standard operating procedure in laboratories, could not be performed by study participants, i.e. hospital nurses on active duty. To minimize the degradation of metabolites by urinary microorganisms or enzymes, the time between donation and storage was strictly specified. Donated samples had to be stored on cool packs (~9°C) by the participants, were collected after the end of the shift by the study nurse and further stored at -80°C. Although the participants were thoroughly briefed about the importance of consistent storage conditions, there was a possibility that some urine samples were stored for a maximum of 24 hours at room temperature. To consider the impact of storage on urinary metabolite profile assessed by targeted metabolomics, which had not been yet investigated, we have therefore conducted a separate study (see *article 1*).

Out of the analyzed and discussed metabolites, we found that the concentration of Serine was significantly affected in both studies. Serine at 20°C showed significantly decreased concentrations when stored for 24 hours compared to those which were immediately frozen (15). As could be seen from *article 2*, among others the amino acid Serine showed increased concentrations during night shift when looking at the full model for creatinine-normalized values (16). The remaining metabolites were relatively stable under various storage conditions. A potential consequence of an inconsistent storage of the urine samples collected for *article 2* would be decreased concentration of the above-mentioned amino acid. Yet, as night shift associated with increased metabolite concentration, it can be assumed that the eventual storage bias was minor.

We employed several approaches to increase the quality of our data analysis and interpretation. Due to the relatively low number of metabolites detected in urine (compared to blood), certain common statistical approaches like a cosine analysis could however not be employed. Moreover, the use of three normalization approaches (a still non-resolved issue in urine metabolomics) led sometimes to inconsistent results. In *article 2,* we employed several normalization approaches since this had been suggested to obtain meaningful differences in the analyzed metabolite profiles (62,85). Yet, different normalization strategies affected the metabolite levels considerably and this could also be observed in *article 2* (62). There has been no consensus on the best normalization approach as each offers certain advantages and disadvantages. The research question and technical circumstances thus dictate the choice of the optimal normalization approach. As a measure that is independent of age or muscle mass, osmolality is dependent of potassium and urea levels and thus requires additional measurements that might not be feasible (62,86). Creatinine normalization is a common approach and makes the comparison among reported and published studies easier. Although creatinine measurements are relatively stable, they can be dependent on time of the day, menstrual cycle, muscle mass and age (87–89). The RBN aims to consider each metabolite's excretion separately. As a rather new and rarely used method, it does thus not allow the inter-study comparability. Thus, we decided to interpret and compare our results with published studies based on creatinine-normalization.

As mentioned in *article 2*, we restricted our analysis to morning urine samples only. This conservative approach was taken to minimize several influencing factors. First, the real life setting allowed the nurses to donate spontaneous urine. Depending on the time since the last donation, the metabolite concentrations in the urine would represent a mean level of metabolite and furthermore activity among metabolite pathways which sums up in the urine. Second, the nurses ate various diets before and after urine donations which could influence the metabolite levels in urine. By restricting to morning urine samples, we assured a better interpersonal comparability of metabolite concentrations. Furthermore, the probability that nurses had eaten before the first urine donation after waking up was considered minor. A sensitivity analysis based on the measurements of *all* urine samples was nevertheless conducted and presented to our collaboration partners (“Forschungsbegleitkreis”). It also showed that medium chain acylcarnitines were the most affected metabolite group in early chronotypes working night shifts and in agreement with the results presented in *article 2*.

Cosine analysis is a common statistical approach for the analysis of changing metabolite concentrations over the course of several hours (56,61). This method assumes a curve-like changes in the metabolite values throughout the day (e.g. cosine wave / 24h) (61). The potential phase shift and amplitude of minimum and maximum metabolite levels per day is then compared between groups (90). Although widely used, this method has several drawbacks. First, our sensitivity analyses showed that when plotting all consecutive metabolite values per person, only a very few metabolites showed a visible cosine rhythm. Moreover, it often did not overlap between individuals or metabolites. Furthermore, implementing mean values would lead to loss of information. Finally, when calculating the phase shift, interpersonal differences could represent a substantial amount of the potential changes (91). For this reason we used linear models in our study.

Since the publication of *article 1 and 2*, more research has been added to the research field of metabolomics and shift work. Homan et al utilized targeted metabolomics to assess the impact of sleep deprivation on certain metabolites and related (92). Urinary metabolite levels of melatonine were related to both rotating shift and effects of shift work on the melatonine levels (93). Furthermore, Kervezee et al could identify several metabolites which were affected in their concentrations during simulated night shifts (90). Gordon-Dseagu et al found sleep to be associated

with metabolites related to obesity (94,95). Interestingly, Dyar et al stressed on the impact of food consumption as a factor of circadian entrainment. They found that in mice on half fat diet, the natural oscillation of NAD+ was degraded, resulting in lower levels (96).

Although various aspects like the sleep related effect on the metabolic profile or the modulation effect of shift work on disease risks are already known through our research, we added the aspect of the individual chronotype when looking at metabolic changes affected by shift work (8,55). Further steps towards the thorough understanding of the "metabolic link" between these two aspects were done through the presented research. First deductions like the consideration of the individual chronotype can immediately be implemented in the planning of shift schedules. One of the important aspects of this work is to provide the groundwork for paving the way to the long term development of potential diagnostic biomarkers. The identified metabolites provide a first selection of metabolites to investigate in more detail, potentially in animal models or via research in a stricter controlled environment.

Still, there are many questions waiting to be answered. To which extent is a broader population in other occupations, including men, affected by night shift or even by working prolonged hours in winter? Can the unfavorable metabolite profile be minimized by customized artificial light with wavelengths that are less disruptive for melatonin production? Which other metabolite classes and biochemical pathways are affected by night shift work? The availability and integration of "big data", also known as omics data, will play a crucial role in the future of circadian occupational medicine. It is my hope that we will soon witness a world where the employers will offer a customized chronotype screening, personalized workday schedule and flexibility as well as regular multi-omic follow up of night shift workers to minimize the impact on their health and increase their life quality and satisfaction.

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Article 1

Stability of targeted metabolite profiles of urine samples under different storage conditions

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ORIGINAL ARTICLE

Stability of targeted metabolite profiles of urine samples under different storage conditions

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Abstract

Introduction Few studies have investigated the influence of storage conditions on urine samples and none of them used targeted mass spectrometry (MS).

Objectives We investigated the stability of metabolite profiles in urine samples under different storage conditions using targeted metabolomics.

Methods Pooled, fasting urine samples were collected and stored at −80 °C (biobank standard), −20 °C (freezer), 4 °C (fridge), ~9 °C (cool pack), and ~20 °C (room temperature) for 0, 2, 8 and 24 h. Metabolite concentrations were quantified with MS using the AbsoluteIDQ™ p150 assay. We used the Welch-Satterthwaite-test to compare the concentrations of each metabolite. Mixed effects linear regression was used to assess the influence of the interaction of storage time and temperature.

Results The concentrations of 63 investigated metabolites were stable at −20 and 4 °C for up to 24 h when compared to samples immediately stored at −80 °C. When stored at ~9 °C for 24 h, few amino acids (Arg, Val and Leu/Ile) significantly decreased by 40% in concentration ($P < 7.9E{-}04$); for an additional three metabolites (Ser, Met, Hexose H1) when stored at ~20 °C reduced up to 60% in concentrations. The concentrations of four more metabolites (Glu, Phe, Pro, and Thr) were found to be significantly influenced when considering the interaction between exposure time and temperature.

Conclusion Our findings indicate that 78% of quantified metabolites were stable for all examined storage conditions. Particularly, some amino acid concentrations were sensitive to changes after prolonged storage at room temperature. Shipping or storing urine samples on cool packs or at room temperature for more than 8 h and multiple numbers of freeze and thaw cycles should be avoided.

Keywords Urine · Storage conditions · Targeted metabolomics · Pre-analytics · Amino acids

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1 Introduction

The field of metabolomics has garnered much attention in recent years (Beger et al. 2016; Bouatra et al. 2013). Potential biomarkers for diseases such as type 2 diabetes and metabolite signatures of medication use and lifestyle choices (e.g. smoking) have been identified (Adam et al. 2016; Brandmaier et al. 2015; Wang-Sattler et al. 2012; Xu et al. 2013, 2015). Identified metabolites provide insight into key physiological mechanisms and underlying pathways.

Extensive research on the influence of storage conditions on human plasma and serum metabolite profiles has been conducted (Breier et al. 2014; Anton et al. 2015). A recent study found that serum concentration of 24 out of 127 quantified metabolites significantly changed at room temperature when compared to the values samples stored at −80 °C (Anton et al. 2015). Regarding plasma, the concentration of 44 out of 159 metabolites changed significantly when kept for 24 h at room temperature (Breier et al. 2014).

With respect to its noninvasiveness, metabolomics of urine samples has become a major focus. The collection of such samples can be conducted without supervision of medical experts (Gao 2013). On the other hand, this means that the samples are not necessarily taken under a controlled, well-regulated clinical environment that enhances measurement reproducibility. Therefore, it is pivotal to determine the effects of pre-analytical sample handling, including storage conditions. Previous research on the pre-analytical effects on human urine samples was regarding non-targeted mass spectrometry (MS) or nuclear magnetic resonance (NMR) technology (Barton et al. 2008; Bernini et al. 2011; Budde et al. 2016; Emwas et al. 2015; Gika et al. 2008; Lauridsen et al. 2007; Roux et al. 2015).

In our study, we use a targeted MS approach to investigate the effects of storage conditions, as well as the interaction of storage time and temperature, on urine metabolite concentrations. The urine samples were stored for 2, 8, and 24 h at temperatures ranging from −80 to ∼20 °C and exposed to up to three freeze–thaw cycles.

2 Materials and methods

2.1 Urine sample preparation under different storage conditions

Urine was collected from six healthy female volunteers between 8:00 and 8:45 am after overnight fasting in sterile disposable containers. To lessen inter-individual differences in urine metabolic profiles, equal parts of urine of each participant were pooled in a sterile 200 ml Erlenmeyer flask and 65 times 1 ml of urine was aliquoted to separate 1.5 ml Eppendorf tubes. Five of these aliquots were immediately frozen at −80 °C and used as baseline reference.

With respect to temperature, we exposed the samples to: (1) room temperature (∼20 °C); (2) cool packs (∼9 °C); (3) fridge (4 °C) and; (4) freezer (−20 °C). Additionally, with respect to the duration, we stored the samples for 2, 8 and 24 h. This resulted in 12 different conditions. As we prepared four biological replicates, a total of 48 further aliquots were therefore used (Fig. 1).

Additionally, the influence of freezing and thawing urine samples was simulated on the remaining 12 aliquots. Urine was frozen for 24 h at −80 °C and thawed for 2 h at ∼20 °C. This cycle was repeated three times (Fig. 1).

2.2 Targeted metabolite quantification

Each sample was measured with the Absolute*IDQ*™ p150 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) and FIA-ESI-MS/MS (flow injection-electrospray ionisation-triple quadrupol mass spectrometry). The assay procedures of the Absolute*IDQ*™ p150 Kit have been described in full detail previously (Römisch-Margl et al. 2012). Samples were prepared by a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK), beside standard laboratory equipment. Mass spectrometric (MS) analyses were done on an API 4000 LC–MS/MS System (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.1. Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the Met*IDQ*™ software package, which is an integral part of the Absolute*IDQ*™ Kit. Metabolite concentrations [μM] were calculated referring to internal standards.

Of 10 μL urine, 162 metabolites were quantified. The baseline reference was measured five times to calculate the coefficients of variance (CV, Table 1). In the course of quality control, we excluded metabolites with a CV higher than 25%. Furthermore, to assure detectability we excluded metabolites with more than 50% of measured values below the limit of detection (three times the median value of water based zero-samples). In total 63 metabolites passed the quality control: free carnitine, 34 acylcarnitines (Cx:y), 13 proteinogenic amino acids, creatinine, hexoses (sum of hexoses), 8 glycerophospholipids (7 phosphatidylcholines (PC) and one lysoPC), and 5 sphingolipids (SM). The abbreviations Cx:y depicts the total number of carbons and

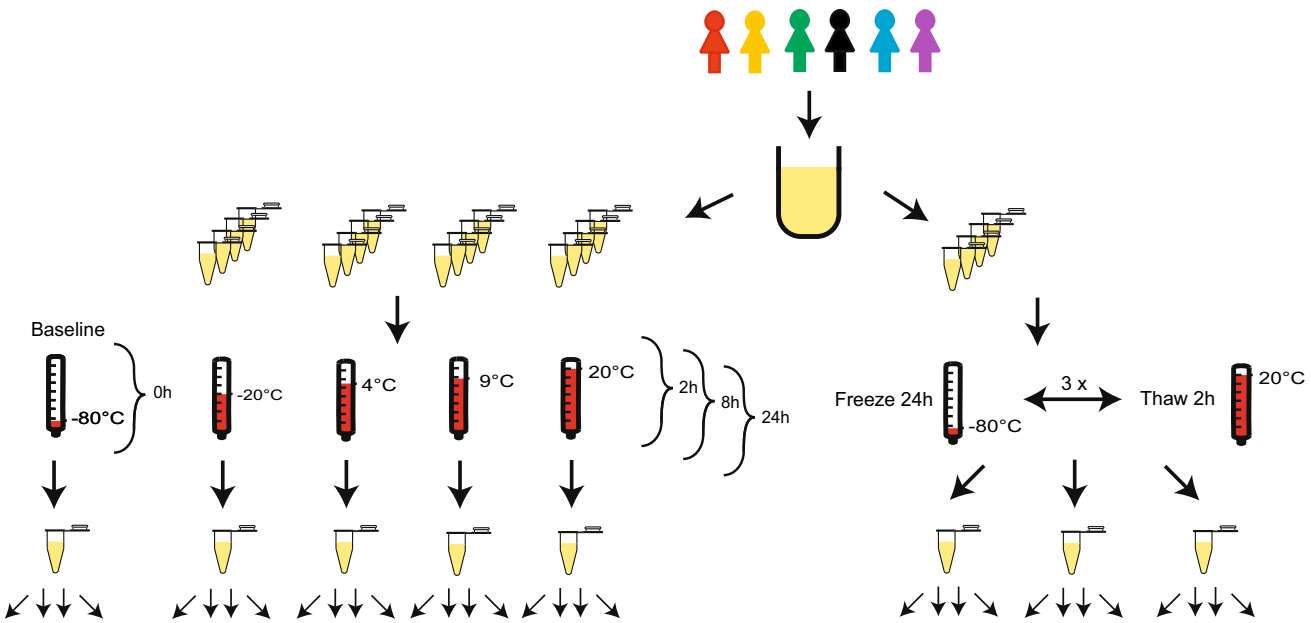

Fig. 1 Overview of the study design. Urine from six female volunteers was pooled and aliquoted before being stored at −80, −20, 4, 9, and 20 °C for 0, 2, 8, and 24 h. For freeze and thaw cycles, samples were frozen for 24 h and thawed for 2 h per cycle. Each sample was measured four times

double bonds of all chains, respectively (for more details see the list of metabolites in Table S1).

2.3 Statistical analysis

To account for technical variation due to measurements, each of the 12 replicates and the three samples that underwent freeze and thaw cycles was measured four times. The resulting values were used to calculate the CV under each condition (Table 1).

All metabolite concentrations were log-transformed and standardized (mean = 0 and standard deviation = 1). For each metabolite under each condition, we performed pairwise comparisons by applying a Welch-Satterthwaite

Table 1 Significant metabolites identified by the pairwise comparison of baseline concentrations against 24 h at ~20 and ~9 °C

Metabolite	Baseline (0 h)	2 h			8 h			24 h		
	Mean (μM)	Change [%]	*P* value	CV (%)	Change (%)	*P* value	CV (%)	Change (%)	*P* value	CV (%)
~20 °C										
Arg	17.40	1.0	0.82	5.8	−28.3	0.03	17.9	−40.9	**3.1E−4**	10.8
Met	12.65	−7.1	0.26	5.1	−15.3	0.16	18.0	−43.7	**1.3E−4**	3.5
Ser	248.63	−3.7	0.44	4.8	−17.4	0.05	12.3	−36.2	**4.0E−5**	6.6
Val	30.86	−4.9	0.43	5.8	−21.7	0.09	20.3	−59.0	**1.0E−5**	3.5
xLeu	50.39	−6.1	0.25	3.0	−22.1	0.07	19.4	−39.6	**8.8E−5**	3.6
H1	622.83	3.7	0.19	2.2	−3.0	0.67	15.0	−30.3	**9.5E−6**	4.3
~9 °C										
C6:1	0.16	1.9	0.77	9.2	2.2	0.76	10.3	16.2	**8.1E−5**	2.6
Arg	17.40	−0.9	0.91	11.0	6.6	0.29	6.3	−40.2	**7.2E−5**	7.0
Val	30.86	−4.9	0.41	4.4	−2.8	0.69	8.8	−42.5	**4.8E−5**	8.1
xLeu	50.39	−2.9	0.64	7.1	0.2	0.96	8.7	−35.3	**1.1E−4**	4.9

The first column shows metabolites with significant changes in their concentration due to storage conditions when compared to the reference samples that are shown in the second column. The following columns show the percentage of concentration change, the respective *P* value, and coefficient variance (CV) derived with from the four measurements of the samples stored at the specified temperatures/conditions (2, 8, and 24 h at ~20 and ~9 °C). Significant *P* values ($P < 7.9E{-}4$) are indicated in bold

separate-variance t test, to assess the differences in metabolite concentration between samples exposed to 2, 8, and 24 h at the respective storage condition with baseline samples (immediately frozen at −80 °C). The same t test was used to pair-wisely compare metabolite concentrations after the freeze and thaw cycles with baseline. To account for multiple testing, Bonferroni correction ($P < 7.9E{-}4 = 0.05/63$) was applied due to 63 used metabolites.

A mixed effects linear regression model was utilized to assess the influence of time and temperature, as well as their interaction term on metabolite concentrations. Additionally, the variables time and temperature were standardized (mean = 0 and standard deviation = 1). The metabolite concentration was used as the dependent variable, time, temperature and their interaction term as the fixed effect and the repeated measurement as random effect.

The mixed effects linear regression model was used to estimate the impact of the number (0–3) of freeze–thaw cycles on each of the used 63 metabolite concentrations.

Statistical analyses were performed with SAS 9.4 (SAS Institute, Cary NC) using '*PROC TTEST*' for pairwise comparisons of storage conditions and '*PROC MIXED*' for the mixed effect linear regression models on the effect of time, temperature, interaction of time, and temperature and number of freeze and thaw cycles.

3 Results

3.1 Amino acids are mostly affected by the storage conditions

We observed that the concentrations of about 90% of examined metabolites in the urine samples were not significantly affected by any of the applied storage conditions (for 0, 2, 8, and 24 h at −20, 4, ∼9, and ∼20 °C, respectively) when compared to samples immediately stored at −80 °C. Only seven out of 63 metabolite concentration measurements were significantly altered. The concentrations of three amino acids were decreased by 35–43% when storing in ∼9 °C, and five by up to about 60% when storing the urine samples at room temperature for 24 h (Table 1). No significant changes in the concentration of any metabolite could be observed for the storage at 4 and −20 °C, when compared to baseline. Furthermore, at ∼20 °C, no changes in the concentration of the examined 63 metabolites could be observed after 2 and 8 h, but at 24 h Arg, Met, Ser, Val, Leu/Ile, and H1 showed a significant decrease (Table 1). We observed a significant decrease for Arg, Val and Leu/Ile and a significant increase for hexenoylcarnitine (C6:1) at ∼9 °C at 24 h (Fig. 2).

3.2 The interaction of temperature and storage time is the most important influence

To further investigate the influence of the combination of time and temperature on the metabolite concentrations, we applied linear mixed effect models. We observed that the concentrations of ten metabolites (Arg, Glu, Met, Phe, Pro, Ser, Thr, Val, Leu/Ile, and H1) showed significant [$P < 2.6E{-}4 = 0.05/(63 \times 3)$ to account for three independent variables and 63 metabolites] associations with the interaction term of time and temperature. These ten metabolites included all that were detected with the pairwise comparison of baseline with samples stored at ∼20 °C for 24 h (Table 2).

3.3 Frequent freeze and thaw cycles influences the sample quality

To investigate the effect of freeze and thaw cycles, we conducted pairwise comparisons between samples that underwent up to three cycles with baseline. We did not observe any significantly changed metabolite concentration for one or two freeze and thaw cycles, but two metabolites (H1 and C3) showed significantly increased concentrations for three freeze and thaw cycles, when compared to samples immediately frozen at −80 °C (Table 3). Hexose H1 concentrations increased gradually from baseline (622.83 µM) starting with cycle one (652.28 µM/4.7%) and two (698.83 µM/12.2%) until 748.95 µM/20.2% increase after the third cycle (Table S2). Results depicting the influence of freeze and thaw cycles on all 63 used metabolites are shown in Table S2.

With the linear mixed effect model, we detected a significant association between the number of freeze and thaw cycles and the concentration of four Acylcarnitines (C3, C4, C8:1, C16:1-OH) and Hexose (Table 4).

4 Discussion

We investigated the influence of storage conditions (temperature, time and freeze and thaw cycles) on metabolite profiles in human urine samples using targeted MS and observed that a full day of storing at room temperature or on cool packs significantly altered the concentration of several metabolites, in particular amino acids. This finding was confirmed by investigating the interaction between exposure time and temperature. Furthermore, we observed that more than two freeze and thaw cycles affected the metabolite concentrations in the urine samples. However, about 78% of quantified metabolites in urine samples from overnight fasting females were not influenced by the

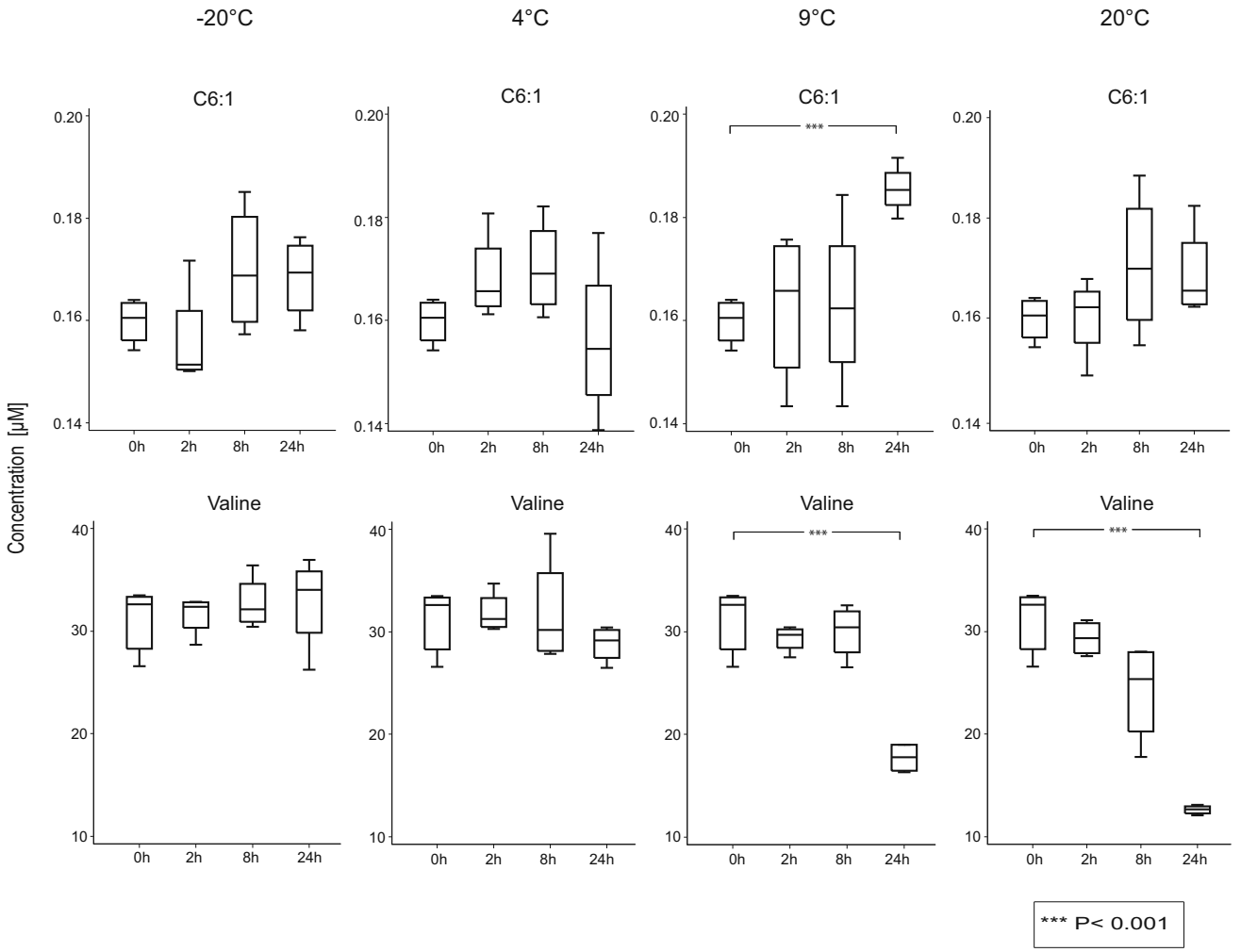

Fig. 2 Concentrations of two metabolites over time for various storage conditions. Influence of storage conditions on the concentrations of Valine and C6:1. The concentration of C6:1 at 9 °C increased, whereas the concentration of valine decreased at 20 and 9 °C in urine over the course of 24 h

examined storage conditions, when considering a stringent Bonferroni corrected level of significance.

Although other studies investigated the impact of storage conditions on metabolite profiles in urine as well, they were using either non-targeted MS or NMR technology (Table 5). Moreover, none of these studies investigated the interaction between storage time and temperature, which previously was only subject to research on the storage of serum samples (Anton et al. 2015).

Our finding of decreased concentrations of Hexose in urine at a prolonged exposure to room temperature is consistent with previous studies in plasma and most likely results from active glycolysis enzymes in urine (Breier et al. 2014; Bruns and Knowler 2009; Grötsch et al. 1985). Furthermore, our observation of decreased concentrations of arginine and methionine in urine is consistent with previous observations in plasma at room temperature (Breier et al. 2014).

The decreased concentrations of the branched chain amino acids (BCAA, i.e. valine, leucine and isoleucine) might be explained by the catabolic activity of a multiple enzyme complex, in particular the branched-chain α-keto acid dehydrogenase (BCKDC). BCKDC converts all three amino acid by: (1) transamination; (2) oxidative decarboxylation; and (3) dehydrogenation (Tanaka and Rosenberg 1983). Indo et al. report BCKDC to be associated with the mitochondrial inner membrane (Indo et al. 1987). Prolonged exposure of urine to room temperature might lead to a degradation of cells and a release of BCKDC. The reduction in the concentration of these BCAA, when stored on cool packs is less profound, when compared to room temperature. The comparably smaller effect is most likely due to the reduced temperature (Gillim et al. 1983).

Contradictory findings were reported for blood: the concentrations of leucine, isoleucine and serine in plasma were found to be increased when stored for 24 h at room

Table 2 Metabolites significantly influenced by the interaction of exposure time and temperature

Metabolite	β-estimate (95% CI)	*P* value
Arg	−0.89 (−1.26, −0.52)	**2.2E−5**
Gln	−0.68 (−0.97, −0.40)	**2.5E−5**
Met	−1.00 (−1.31, −0.70)	**1.2E−7**
Phe	−0.71 (−1.03, −0.39)	**7.8E−5**
Pro	−0.83 (−1.20, −0.46)	**5.8E−5**
Ser	−1.01 (−1.26, −0.75)	**9.8E−10**
Thr	−0.69 (−1.01, −0.38)	**7.7E−5**
Val	−1.01 (−1.44, −0.58)	**2.8E−5**
xLeu	−0.95 (−1.28, −0,61)	**1.2E−6**
H1	−1.14 (−1.51, −0.76)	**4.5E−7**

The first column shows metabolites with a significant influence on the interaction of time and temperature. The following columns show β-estimates with respective confidence intervals (95%) and *P* values via a linear mixed effects model. Significant *P* values ($P < 2.64E{-}4$) are indicated in bold

Table 4 Metabolites significantly associated with the number of freeze and thaw cycles

Metabolite	β-estimate (95% CI)	*P* value
C3	0.53 (0.41–1.03)	**2.5E−4**
C4	0.62 (0.36–0.88)	**1.8E−4**
C8:1	0.70 (0.39–1.01)	**2.9E−4**
C16:1-OH	0.72 (0.41–1.03)	**2.5E−4**
H1	0.45 (0.27–0.64)	**1.5E−4**

The first column shows metabolites that were significantly associated with the number of freeze and thaw cycles. The following columns show β-estimates with respective confidence intervals (95%) and *P* values derive with a linear mixed effects model. Significant *P* values ($P < 7.9E{-}4$) are indicated in bold

temperature and for leucine when stored for 24 h on cool packs (Breier et al. 2014). However this is expected, when comparing different matrices, such as blood and urine. Urine at prolonged room temperature is prone to getting more acidic, whereas blood is buffered (Alguacil et al. 2007). Additionally, the number of cells is not comparable between the two matrices, which also accounts for the protein concentration and numerous other factors, such as bacterial growth. To avoid such interferences by reducing the number of bacteria and host cells, as well as large particles, previous studies suggested to pre-centrifuge (1000–3000 RCF for 5 min at 4 °C) and filter urine samples (using a 0.20 µm filter) before conducting the metabolite profiling (Bernini et al. 2011; Emwas et al. 2015). However, certain circumstances like the non-availability of filters and centrifuges and time consuming operation during real life studies make it difficult to use said filters directly after donation.

We observed up to 60% reduction of BCAA (isoleucine and valine) when urine samples are stored on cool packs or at room temperature for 24 h. These BCAA are in particular essential for the growth of certain microorganisms, such as *lactobacillus brevis* and *lactobacillus plantarum* (Katina 2005). *L. brevis* can be found in intestines, colon and vagina (Makarova et al. 2006). The interplay of time and temperature may have led to a consumption and consequent concentration reduction of the respective amino acids in urine samples.

The detected increase in Hexose (mainly glucose) concentration after multiple freeze and thaw cycles might be due to the reported degeneration of sucrose. The enzyme invertase, also called sucrase, catalyzes the hydrolysis of sucrose to glucose and fructose (Huang et al. 1999; Zhang et al. 2016). Invertase is found in the potential urine contaminant yeast (Fisher et al. 1995). Repeated freezing and thawing might have damaged these cells, led to a diffusion of invertase in urine and consequently the hydrolysis of sucrose. Additionally, the acid-catalyzed hydrolysis of sucrose was reported to be enhanced by freezing (Lund et al. 1969). Both mechanisms are likely to explain the gradual increase of glucose concentrations (up to 20%) after the freeze and thaw cycles.

However, the observation of an opposite trend for the glucose concentration after freeze and thaw cycles, when compared to the storing at room temperature for 24 h needs to be further investigated.

In general, using targeted MS approach we observed changes in six metabolites (C3, C6:1, Arg, Val, Leu/Iso

Table 3 Metabolites significantly influenced by freeze and thaw cycles

Metabolite	Baseline (0 h)	Cycle 1 (26 h)			Cycle 2 (52 h)			Cycle 3 (78 h)		
	Mean (µM)	Change (%)	*P* value	CV (%)	Change (%)	*P* value	CV (%)	Change (%)	*P* value	CV (%)
C3	1.00	1.3	0.55	2.3	7.7	0.13	7.1	13.8	**7.7E−4**	1.3
H1	622.84	4.7	0.24	5.6	12.2	0.07	8.3	20.2	**3.1E−4**	3.4

The first column shows metabolites that were significantly changed after the third freeze and thaw cycle when compared to the baseline values that are shown in the second column. The following columns show the percentage of concentration change, the respective *P* value, and coefficient variance (CV) derived with from the measurements of the sample after one, two, and three freeze and thaw cycles. Significant *P* values ($P < 7.9E{-}4$) are indicated in bold

Table 5 Previous studies on pre-analytical sample handling

Author	Technique	Approach	Tissue	Temperature	Time	Interaction	Freeze/thaw cycles
Lauridsen et al. (2007)	NMR	Non-targeted	Urine	−80, −25, and 4 °C	0, 1, 2, 3, 4, 6, 10, 14, and 26 weeks	–	–
Barton et al. (2008)	NMR	Non-targeted	Urine, serum	−80 and 4 °C	0, 24, and 36 h	–	–
Bernini et al. (2011)	NMR	Non-targeted	Urine, serum plasma	−80, 4 °C, and RT	0, 2, 4, 6, 24 h, and 1 week	–	–
Roux et al. (2015)	NMR, MS	Non-targeted	Urine	−80, 4 °C, and RT (19–26 °C)	0–72 h (every 4 or 12 h)	–	–
Budde et al. (2016)	NMR	Non-targeted	Urine	−80, 4, 10 °C, and RT (25 °C)	0, 1, 2, 8, 10, 12, 24, 28, 72 h, and 1 month	–	–
Gika et al. (2008)	MS	Non-targeted	Urine	−80, −20, and −4 °C,	1 week, 1 month, 3 months, and 6 months	–	X (−20 °C)
Breier et al. (2014)	MS	Targeted	Serum, plasma	−80, ~4 °C, and RT (21 °C)	0, 3, 6, and 24 h	–	X (−20 °C)
Anton et al. (2015)	MS	Targeted	Serum	−80 °C, dry ice, wet ice, and RT (22–24 °C)	0, 12, 24, and 36 h	X	X (−80 °C)
Rotter et al.	MS	Targeted	Urine	−80, −20, 4, ~9 °C, and RT (~20 °C)	0, 2, 8, and 24 h	X	X (−80 °C)

The first three columns indicate the underlying study and the technical approach that was applied. The following columns depict the examined tissue and the pre-processing conditions (temperature, time, and their interaction), the samples were exposed to. The final column indicates if freeze and thaw cycles were subject to the respective study

RT room temperature, *NMR* nuclear magnetic resonance, *MS* mass spectrometry

(xLeu), H1) that were not reported in other studies that applied similar storage conditions (e.g. 24 h at 10 °C, up to nine freeze and thaw cycles), but different measurement techniques (NMR, non-targeted MS) (Budde et al. 2016; Gika et al. 2008). Other observed alterations in the metabolite profiles were derived under conditions that were not part of our study (e.g. 72 h or 12 weeks at 4 °C) (Lauridsen et al. 2007; Roux et al. 2015). Due to the comparably small number of measurements, our study is limited in statistical power. Additionally, the Absolute*IDQ*^{TM} p150 kit was originally developed for blood samples, and not focusing on urine. This is reflected by the number of only 63 metabolites that passed the quality control. In order to make the kit more applicable to urine, creatinine was included by the manufacturer into the metabolite panel, to enable researchers to account and normalize for different urine excretion rates. Furthermore, the measured values were not derived from biological replicates, but from repeated measurements of the same samples. However, by this procedure, potential analytical variations could be identified and accounted for. Furthermore, by using pooled samples, we do not have to account for different excretion rates and differences in interpersonal metabolite profiles. This supports the direct comparability of measured effects.

5 Conclusions

The findings from our study suggested to avoid shipping urine samples on cool packs or at room temperature for durations of more than 8 h, and we have provided insight on improved planning and sample maintenance in the field. We strongly recommend storage temperatures of at least −20 °C and to minimize the number of freeze and thaw cycles to ensure integrity of urine samples used for metabolomics studies.

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Author contributions MR, SB, and RW-S designed the experiments, MR conducted the pre-analytical experiments and analysed the data; CP and JA supervised and performed metabolomics measurements; CP, JA, SR, KG, TB, JADAM, and HL contributed reagents/materials/analysis; MR, SB, and RW-S wrote the paper.

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Compliance with ethical standards

Conflict of interest Markus Rotter, Stefan Brandmaier, Cornelia Prehn, Jonathan Adam, Sylvia Rabstein, Katarzyna Gawrych, Thomas Brüning, Thomas Illig, Heiko Lickert, Jerzy Adamski, Rui Wang-Sattler declare that they have no conflict of interest.

Ethical approval Since there was no identifying information obtained from our participants who donated urine for our study, the 'Bayerische Landesärztekammer' declared that our study was not subject to compliance with ethical standards regarding the use of humans in research.

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Article 2

Night Shift Work Affects Urine Metabolite Profiles of Nurses with Early Chronotype

metabolites

MDPI

Article

Night Shift Work Affects Urine Metabolite Profiles of Nurses with Early Chronotype

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Abstract: Night shift work can have a serious impact on health. Here, we assess whether and how night shift work influences the metabolite profiles, specifically with respect to different chronotype classes. We have recruited 100 women including 68 nurses working both, day shift and night shifts for up to 5 consecutive days and collected 3640 spontaneous urine samples. About 424 waking-up urine samples were measured using a targeted metabolomics approach. To account for urine dilution, we applied three methods to normalize the metabolite values: creatinine-, osmolality- and regression-based normalization. Based on linear mixed effect models, we found 31 metabolites significantly (false discovery rate <0.05) affected in nurses working in night shifts. One metabolite, acylcarnitine C10:2, was consistently identified with all three normalization methods. We further observed 11 and 4 metabolites significantly associated with night shift in early and late chronotype classes, respectively. Increased levels of medium- and long chain acylcarnitines indicate a strong impairment of the fatty acid oxidation. Our results show that night shift work influences acylcarnitines and BCAAs, particularly in nurses in the early chronotype class. Women with intermediate and late chronotypes appear to be less affected by night shift work.

Keywords: metabolomics; urine normalization; women's' health; night shift work; chronotypes

1. Introduction

About one fifth of employees in industrialized countries are working in some type of shift schedule [1]. Shift work was reported to have adverse health effects and increase the chance for diseases like obesity and type 2 diabetes [2–5]. When forced to work at a non-standard time (e.g., night shift), which is not in concordance with the personal inner clock, individuals tend to develop a "social jet lag," a discrepancy between sleep timing on work days and free days [2,6]. The difference in the preferred personal timing or chronotype plays a major role in individual sleep duration and health risk when working night shift [5,7]. Previous research showed that female night shift workers with morning preferences had a higher risk for breast cancer compared to those with evening preferences [3]. The underlying metabolic pathways affected by shift work and the role of the individual chronotype in this respect have not been studied in detail.

Targeted metabolomic profiling in human blood has been used to assess lifestyle or disease effects such as pre-diabetes, sleep curtailment and sleep deprivation [8–13]. However, no real-life study investigating the impact of night shift work on the urine metabolic profile of participants with early, intermediate and late chronotype has been reported. Major advantages of urine bio samples are their non–invasive sampling, moreover, urine samples are well studied with respect to age, obesity and storage conditions [14–17]. However, as urine samples are susceptible to a variety of factors such as water intake, it was suggested to either use alternative methods besides creatinine- or osmolality-normalizations, or to apply more than one normalization method [18–20].

Here, we have used the Munich Chronotype Questionnaire for shift workers ($MCTQ^{Shift}$) to assess the chronotype of participants working in shift work [21]. We collected about 3640 longitudinal multi-time-point urine samples and analysed 424 waking-up samples (a proxy for fasting samples) from 68 female nurses working on several consecutive days during day or night shift. Targeted urine metabolite profiles were normalized with creatinine-, osmolality- and regression based normalization (RBN) and the results allowed us to investigate the influence of night shift on the targeted metabolite profile in urine of female nurses while considering their individual chronotypes.

2. Results

2.1. Characteristics of Participants

We recruited 100 study participants and used 97 women for a chronotype classification (Figure 1). These women had a mean age of 39.5 (25.0–60.0) and an average chronotype of 04:02 (01:17) (Table 1).

Furthermore, we analysed 424 waking-up samples of 68 shift working (SW) nurses. The 68 SW nurses had mean age of 37.2 years (Table 1). We stratified the 68 SW nurses into early (N = 30), intermediate (N = 22) and late chronotype (N = 16) classes (Figure 1). The age of nurses in early and intermediate chronotype classes was comparable whereas nurses in the late chronotype class were younger (Table 1). We observed a negative correlation between age and chronotype values (Pearson correlation coefficient $r = -0.45$) (Figure S1). Moreover, early chronotypes smoked less and reported no respiratory diseases and a lower number of allergies when compared to late chronotypes. Respiratory diseases were most common (24%) among intermediate chronotypes (Table 1).

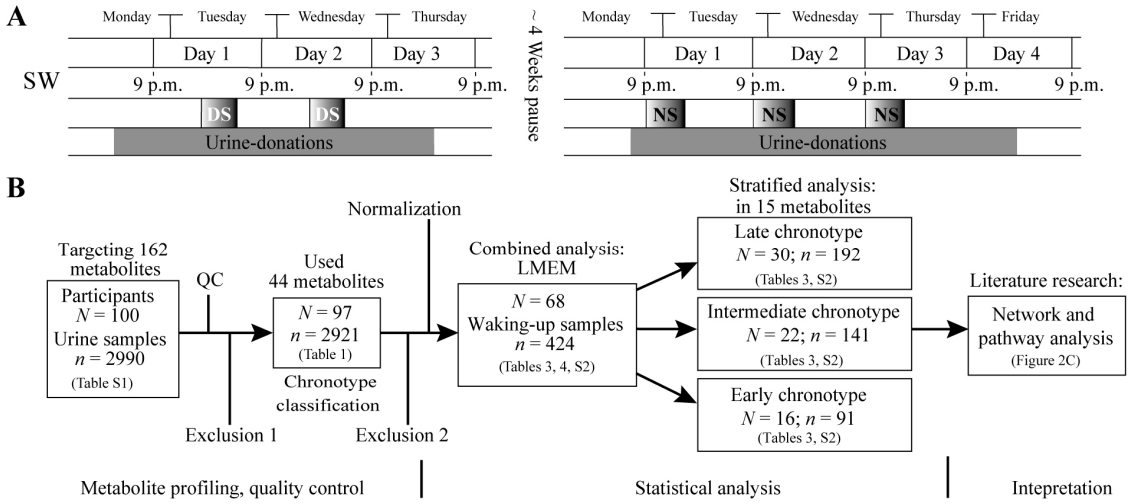

Figure 1. Study design and work flow for night shift and day shift comparison. Plot (**A**) shows an overview for nurses in SW (working both day and night shift) performing two shifts from Monday to Friday in day shift (DS) and night shift (NS) respectively with around a 4 week pause in between the study blocks. Boxes labelled DS indicate working hours in day shift, boxes labelled NS indicate working hours in night shift. Throughout the whole shift and observation period, urine samples were collected (grey boxes). Day 1 to day 4 lasts from 9 p.m. to 9 p.m. the next day and defines the time periods for comparison of day shift and night shift metabolic profiles; Plot (**B**) demonstrates an overview of the urine sample collection and exclusion, as well as the consecutive statistical and pathway analysis. Exclusion 1 = Exclude participants without information on sleep; Exclusion 2 = Exclude diabetics, vegetarians and participants with extreme sleep apnoea, as well as women working only day shift.

Table 1. Characteristics of participants. Characteristics of all participants, shift working group (SW) in combined and stratified analyses are shown. Means with standard deviations (SD) or number of phenotypes with percentages are shown for each group. BMI (body mass index). * Chronotype is defined as mid-sleep corrected for sleep debt accumulated over the past work week.

Clinical Parameters	All Participants	Shift Working Participants (Combined Analysis)	Stratified Analysis		
			Early Chronotype	Intermediate Chronotype	Late Chronotype
N	97	68	16	22	30
Chronotype (SD) *, a.m.	04:02 (01:17)	04:21 (01:14)	02:50 (00:43)	03:59 (00:14)	05:26 (00:48)
Mean age (range), years	39.5 (25.0–60.0)	37.2 (25.0–57.0)	41.3 (25.0–50.0)	40.5 (25.0–57.0)	32.5 (25.0–56.0)
BMI, kg/m^2	26.2 (5.2)	26.2 (5.0)	26.6 (4.5)	26.7 (5.5)	25.7 (5.0)
Regular smoker (%)	27 (27.8)	26 (38.2)	3 (18.8)	10 (45.5)	13 (43.3)
Thyroid disease (%)	20 (20.1)	12 (17.6)	4 (25.0)	2 (9.1)	6 (19.4)
Hypertension (%)	16 (16.5)	10 (14.7)	2 (12.5)	3 (13.6)	5 (16.7)
Respiratory disease (%)	14 (14.4)	9 (13.4)	0 (0.0)	5 (23.8)	4 (13.3)
Cases of Allergy (%)	53 (54.6)	37 (54.4)	6 (37.5)	11 (50.0)	20 (66.7)
Kidney disease (%)	2 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

2.2. Correlation of 44 Metabolites Comparing Three Normalization Methods

Out of 162 quantified metabolites (Table S1), 44 metabolites passed our stringent quality control (see method). When comparing the measured creatinine concentration with the measured osmolality value in the 424 waking-up urine samples, we observed a moderate correlation ($r = 0.72$, p-value = 1.4×10^{-4}). Furthermore, for the used 44 metabolites, we detected various correlation ranges among creatinine-, osmolality- and RBN normalized values (Table 2). The most significant correlation coefficients could be observed between RBN and creatinine-normalized values (r ranged from 0.48 to 0.99). The observed r ranged from 0.35 to 0.93 for the comparison of RBN with osmolality-normalized values and from −0.09 to 0.93 for the comparison between creatinine- and osmolality-normalized values (Table 2).

Table 2. Correlation of 44 metabolites concentrations after creatinine-, osmolality- and regression-based normalization. Pearson correlation coefficients of three pairwise comparisons are shown. Non-significant correlations are indicated in bold (Bonferroni cut off; p-values < 1.14×10^{-3}). In summary, absolute correlation coefficients > 0.15 reflect a statistical significance.

Metabolite	Creatinine Normalization vs. Osmolality Normalization	Osmolality Normalization vs. RBN	RBN vs. Creatinine Normalization
C0	0.76	0.84	0.97
C2	0.84	0.85	0.98
C3	0.68	0.76	0.96
C4:1	0.93	0.93	0.99
C5	0.73	0.89	0.93
C5-M-DC	0.75	0.83	0.96
C5:1	0.62	0.79	0.93
C5:1-DC	0.68	0.76	0.95
C6:1	**0.13**	0.55	0.71
C7-DC	0.21	0.56	0.79
C8	−0.01	0.49	0.61
C8:1	0.70	0.72	0.96
C9	0.65	0.77	0.93
C10	**−0.08**	0.49	0.57
C10:1	0.18	0.49	0.79
C10:2	0.74	0.82	0.96
C12	**0.09**	0.63	0.64
C14	0.16	0.66	0.56
C14:1	0.23	0.70	0.70
C14:1-OH	0.16	0.70	0.61
C14:2	**0.12**	0.64	0.62
C14:2-OH	**0.05**	0.61	0.57
C16	0.47	0.78	0.77
C16-OH	0.68	0.90	0.86
C16:2	0.19	0.75	0.48
C18:2	0.32	0.76	0.49
Arg	0.33	0.44	0.80
Gln	0.44	0.50	0.86
Gly	0.64	0.60	0.92
His	0.58	0.51	0.90
Met	**−0.09**	0.35	0.61
Phe	0.42	0.52	0.87
Pro	0.22	0.42	0.71
Ser	0.44	0.55	0.85
Thr	0.50	0.53	0.89
Trp	0.21	0.41	0.81
Tyr	0.48	0.49	0.88
Val	0.28	0.36	0.75
Leu/Isoleu	0.45	0.36	0.76
Creatinine	-	0.20	-
PC ae C38:3	0.44	0.83	0.61
PC ae C38:6	**0.11**	0.55	0.66
SM C24:0	0.44	0.80	0.72
H1	0.50	0.59	0.88

2.3. Metabolites Associated with Night Shift in the Combined Analysis in Three Normalization Methods

Based on creatinine-normalized values, out of 44 analysed metabolites, urine concentrations of 15 metabolites were significantly altered between night and day shift, both in basic and full models (Table 3 and Table S2). The 15 metabolites consisted of 11 medium- and long-chain acylcarnitines (C5, C7-DC, C8, C10, C10:2, C12, C14, C14:1, C14:1-OH, C14:2, C14:2-OH), three amino acids (phenylalanine, glycine, serine) and one sphingomyelin (SM C24:0). With the exception of C10:2, we observed increased concentrations of the identified acylcarnitines in urine that was donated during night shift, when compared to urine from day shift (Figure 2A, Table 3 and Table S2).

Based on the osmolality-normalization, urine concentrations of 17 metabolites were significantly altered by night shift in both basic and full LMEM models (Table 4). The identified metabolites included six acylcarnitines, six amino acids, creatinine, PC ae C38:3 and hexose H1.

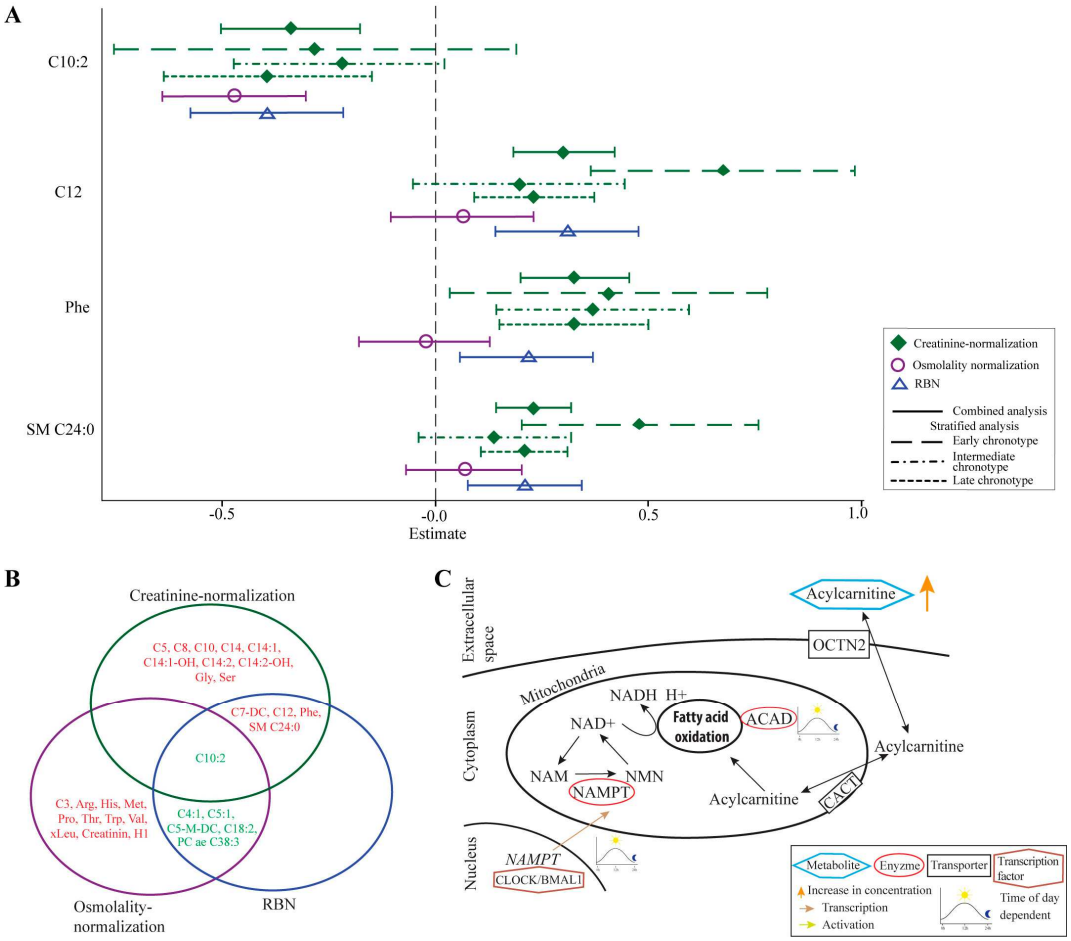

Figure 2. Results of four selected metabolites of three normalization methods of combined and chronotype-stratified analyses as well as pathways potentially affected by night shift work. Plot (**A**) shows the β-estimates and associated 95% confidence intervals of four metabolites based on creatinine-, osmolality- and regression based normalization of combined and stratified analysis; Plot (**B**) shows a Venn diagram of significantly altered metabolites when comparing DS with NS in the combined analysis of three normalization methods. Colours indicate the direction of the observed change (red = increase, green = decrease); Plot (**C**) depicts an overview for pathways potentially affected by night shift work. OCTN2, cell membrane carrier; CACT, Carnitine acylcarnitine translocase; NAD, Nicotinamide adenine dinucleotide (oxidised); NADH, Nicotinamide adenine dinucleotide (deoxidised); NAM, Nicotinamide; NMN, Nicotinamide mononucleotide; NAMPT, nicotinamide phosphoribosyl transferase; *NAMPT*, Gene encoding NAMPT.

Table 3. Results of 15 metabolites significantly altered by night shift work in combined and chronotype-stratified analysis based on creatinine-normalized values in the fully adjusted model. For each metabolite, the β-estimate, the 95% confidence interval (CI) and false discovery rate (FDR) of the full linear mixed effect model (LMEM) for the comparison of day shift (reference) and night shift are shown. The full linear model was adjusted for chronotype, batch effect, smoking status, age, BMI, thyroid disease status, total years of shift work, day of shift and time since last urination. Significant *p*-values (FDR < 0.05) in both basic and full LMEM model are indicated in bold. *N* = Number of nurses; *n* = Number of urine samples.

	Combined Analysis *N* = 68; *n* = 424		**Early Chronotype *N* = 16; *n* = 91**		**Intermediate Chronotype *N* = 22; *n* = 141**		**Late Chronotype *N* = 30; *n* = 192**	
Metabolites	**β–Estimate (95% CI)**	**FDR *p*-value**	**β–Estimate (95% CI)**	**FDR *p*-value**	**β-Estimate (95% CI)**	**FDR *p*-Value**	**β-Estimate (95% CI)**	**FDR *p*-Value**
C5	0.09 (0.03, 0.15)	$\mathbf{1.8 \times 10^{-2}}$	0.24 (0.10, 0.38)	$\mathbf{6.3 \times 10^{-3}}$	−0.02 (−0.12, 0.09)	0.87	0.10 (0.01, 0.19)	0.12
C7-DC	0.23 (0.12, 0.35)	$\mathbf{7.1 \times 10^{-4}}$	0.53 (0.25, 0.81)	$\mathbf{4.3 \times 10^{-3}}$	0.14 (−0.08, 0.35)	0.47	0.15 (−0.01, 0.31)	0.23
C8	0.15 (0.05, 0.26)	$\mathbf{1.7 \times 10^{-2}}$	0.51 (0.23, 0.79)	$\mathbf{4.3 \times 10^{-3}}$	0.00 (−0.21, 0.20)	0.99	0.16 (0.02, 0.29)	0.12
C10	0.18 (0.07, 0.28)	$\mathbf{4.7 \times 10^{-3}}$	0.57 (0.26, 0.88)	$\mathbf{4.3 \times 10^{-3}}$	0.05 (−0.15, 0.25)	0.83	0.14 (0.01, 0.27)	0.12
C10:2	−0.34 (−0.50, −0.18)	$\mathbf{5.1 \times 10^{-4}}$	−0.22 (−0.69, 0.24)	0.46	−0.22 (−0.47, 0.03)	0.38	−0.39 (−0.64, −0.14)	$\mathbf{2.3 \times 10^{-2}}$
C12	0.30 (0.18, 0.42)	$\mathbf{1.4 \times 10^{-5}}$	0.68 (0.37, 0.99)	$\mathbf{2.0 \times 10^{-3}}$	0.19 (−0.06, 0.44)	0.41	0.23 (0.09, 0.37)	$\mathbf{2.3 \times 10^{-2}}$
C14	0.16 (0.04, 0.27)	$\mathbf{2.1 \times 10^{-2}}$	0.53 (0.19, 0.86)	$\mathbf{8.7 \times 10^{-3}}$	0.09 (−0.13, 0.32)	0.66	0.04 (−0.10, 0.19)	0.78
C14:1	0.18 (0.07, 0.28)	$\mathbf{4.7 \times 10^{-3}}$	0.52 (0.23, 0.82)	$\mathbf{4.6 \times 10^{-3}}$	0.14 (−0.06, 0.35)	0.45	0.04 (−0.09, 0.17)	0.78
C14:1-OH	0.21 (0.09, 0.33)	$\mathbf{4.7 \times 10^{-3}}$	0.58 (0.23, 0.93)	$\mathbf{6.6 \times 10^{-3}}$	0.20 (−0.04, 0.43)	0.41	0.05 (−0.10, 0.20)	0.78
C14:2	0.18 (0.07, 0.29)	$\mathbf{6.6 \times 10^{-3}}$	0.50 (0.17, 0.82)	$\mathbf{1.1 \times 10^{-2}}$	0.13 (−0.07, 0.34)	0.47	0.08 (−0.06, 0.22)	0.52
C14:2-OH	0.16 (0.05, 0.28)	$\mathbf{1.8 \times 10^{-2}}$	0.56 (0.22, 0.91)	$\mathbf{7.6 \times 10^{-3}}$	0.09 (−0.13, 0.30)	0.66	0.04 (−0.10, 0.18)	0.81
Gly	0.16 (0.05, 0.28)	$\mathbf{1.8 \times 10^{-2}}$	−0.09 (−0.35, 0.17)	0.65	0.27 (0.07, 0.46)	0.10	0.21 (0.04, 0.39)	0.12
Phe	0.33 (0.20, 0.45)	$\mathbf{1.4 \times 10^{-5}}$	0.40 (0.03, 0.78)	8.4×10^{-2}	0.37 (0.14, 0.60)	6.9×10^{-2}	0.32 (0.15, 0.50)	$\mathbf{8.0 \times 10^{-3}}$
Ser	0.15 (0.03, 0.27)	$\mathbf{3.8 \times 10^{-2}}$	−0.06 (−0.35, 0.23)	0.77	0.22 (0.02, 0.43)	0.29	0.17 (−0.02, 0.36)	0.25
SM C24:0	0.23 (0.14, 0.32)	$\mathbf{1.4 \times 10^{-5}}$	0.48 (0.20, 0.76)	$\mathbf{5.3 \times 10^{-3}}$	0.14 (−0.04, 0.32)	0.41	0.21 (0.11, 0.31)	$\mathbf{4.1 \times 10^{-3}}$

Table 4. Metabolites associated with night shift (osmolality- and regression-based normalized values). The table shows β-estimates and false discovery rate (FDR) values for 21 metabolites which are significantly associated with night shift work. The calculations were based on osmolality and regression-based normalizations. The basic LMEM was adjusted for chronotype value and batch effect. The full model was adjusted for chronotype value, batch effect, BMI, age, smoking status, thyroid disease status, total years of shift work, day of shift and time since last urination. Significant FDR values are indicated in bold.

Metabolite	Osmolality-Normalization				Regression Based Normalization			
	Basic Model		Full model		Basic Model		Full model	
	β-Estimate (95% CI)	FDR	β-Estimate (95% CI)	FDR	β-Estimate (95% CI)	FDR	β-Estimate (95% CI)	FDR
C3	−0.23 (−0.39, −0.07)	$\mathbf{2.0 \times 10^{-2}}$	−0.25 (−0.42, −0.07)	$\mathbf{1.9 \times 10^{-2}}$	−0.14 (−0.29, 0.02)	0.17	−0.15 (−0.32, 0.01)	0.17
C4:1	−0.10 (−0.16, −0.04)	$\mathbf{3.7 \times 10^{-3}}$	−0.10 (−0.16, −0.05)	$\mathbf{4.2 \times 10^{-3}}$	−0.07 (−0.12, −0.02)	$\mathbf{2.1 \times 10^{-2}}$	−0.07 (−0.12, −0.02)	$\mathbf{4.5 \times 10^{-2}}$
C5-M-DC	−0.26 (−0.37, −0.14)	$\mathbf{2.5 \times 10^{-4}}$	−0.29 (−0.41, −0.16)	$\mathbf{1.1 \times 10^{-4}}$	−0.22 (−0.32, −0.11)	$\mathbf{5.9 \times 10^{-4}}$	−0.24 (−0.35, −0.13)	$\mathbf{4.6 \times 10^{-4}}$
C5:1	−0.27 (−0.42, −0.12)	$\mathbf{3.5 \times 10^{-3}}$	−0.32 (−0.48, −0.16)	$\mathbf{9.2 \times 10^{-4}}$	−0.19 (−0.33, −0.06)	$\mathbf{2.1 \times 10^{-2}}$	−0.25 (−0.39, −0.11)	$\mathbf{4.7 \times 10^{-3}}$
C7-DC	−0.05 (−0.21, 0.11)	0.62	−0.04 (−0.21, 0.14)	0.72	0.26 (0.10, 0.42)	$\mathbf{1.1 \times 10^{-2}}$	0.29 (0.12, 0.46)	$\mathbf{6.9 \times 10^{-3}}$
C10:2	−0.49 (−0.65, −0.33)	$\mathbf{1.6 \times 10^{-7}}$	−0.47 (−0.64, −0.30)	$\mathbf{4.3 \times 10^{-6}}$	−0.42 (−0.58, −0.25)	$\mathbf{2.0 \times 10^{-5}}$	−0.39 (−0.57, −0.22)	$\mathbf{4.1 \times 10^{-4}}$
C12	0.03 (−0.12, 0.19)	0.72	0.06 (−0.11, 0.23)	**0.51**	0.25 (0.09, 0.41)	$\mathbf{1.2 \times 10^{-2}}$	0.31 (0.14, 0.48)	$\mathbf{3.5 \times 10^{-3}}$
C18:2	−0.19 (−0.34, −0.04)	$\mathbf{3.5 \times 10^{-2}}$	−0.23 (−0.39, −0.07)	$\mathbf{1.8 \times 10^{-2}}$	−0.25 (−0.40, −0.09)	$\mathbf{1.1 \times 10^{-2}}$	−0.32 (−0.48, −0.15)	$\mathbf{1.7 \times 10^{-3}}$
Arg	−0.29 (−0.44, −0.15)	$\mathbf{8.8 \times 10^{-4}}$	−0.31 (−0.46, −0.16)	$\mathbf{9.2 \times 10^{-4}}$	−0.20 (−0.36, −0.04)	6.2×10^{-2}	−0.26 (−0.43, −0.08)	$\mathbf{2.1 \times 10^{-2}}$
His	−0.15 (−0.28, −0.03)	$\mathbf{3.8 \times 10^{-2}}$	−0.16 (−0.29, −0.03)	$\mathbf{4.3 \times 10^{-2}}$	−0.03 (−0.15, 0.08)	0.70	−0.05 (−0.17, 0.08)	0.56
Met	−0.23 (−0.38, −0.07)	$\mathbf{1.7 \times 10^{-2}}$	−0.24 (−0.40, −0.08)	$\mathbf{1.4 \times 10^{-2}}$	−0.13 (−0.30, 0.03)	0.21	−0.16 (−0.33, 0.02)	0.18
Phe	0.00 (−0.15, 0.14)	0.98	−0.02 (−0.18, 0.13)	0.76	0.24 (0.09, 0.39)	$\mathbf{1.1 \times 10^{-2}}$	0.21 (0.06, 0.37)	$\mathbf{3.6 \times 10^{-2}}$
Pro	−0.24 (−0.38, −0.09)	$\mathbf{9.9 \times 10^{-3}}$	−0.25 (−0.41, −0.10)	$\mathbf{7.0 \times 10^{-3}}$	−0.05 (−0.19, 0.09)	0.61	−0.09 (−0.24, 0.06)	0.36
Thr	−0.20 (−0.36, −0.05)	$\mathbf{3.2 \times 10^{-2}}$	−0.22 (−0.38, −0.06)	$\mathbf{2.7 \times 10^{-2}}$	−0.09 (−0.23, 0.05)	0.42	−0.12 (−0.27, 0.04)	0.28
Trp	−0.19 (−0.32, −0.06)	$\mathbf{2.0 \times 10^{-2}}$	−0.21 (−0.36, −0.07)	$\mathbf{1.3 \times 10^{-2}}$	−0.01 (−0.12, 0.11)	0.90	−0.05 (−0.17, 0.07)	0.56
Val	−0.18 (−0.33, −0.03)	$\mathbf{4.1 \times 10^{-2}}$	−0.20 (−0.36, −0.04)	$\mathbf{4.1 \times 10^{-2}}$	−0.07 (−0.22, 0.09)	0.56	−0.11 (−0.28, 0.05)	0.31
Leu/Isoleu	−0.25 (−0.40, −0.10)	$\mathbf{7.6 \times 10^{-3}}$	−0.27 (−0.43, −0.11)	$\mathbf{5.1 \times 10^{-3}}$	−0.09 (−0.24, 0.07)	0.49	−0.13 (−0.30, 0.04)	0.28
Creatinine	−0.18 (−0.33, −0.04)	$\mathbf{3.4 \times 10^{-2}}$	−0.19 (−0.34, −0.04)	$\mathbf{3.5 \times 10^{-2}}$	−0.15 (−0.30, 0.00)	0.12	−0.18 (−0.34, −0.02)	$\mathbf{9.3 \times 10^{-2}}$
PC ae C38:3	−0.35 (−0.51, −0.18)	$\mathbf{6.8 \times 10^{-4}}$	−0.34 (−0.51, −0.16)	$\mathbf{2.1 \times 10^{-3}}$	−0.57 (−0.74, −0.40)	$\mathbf{4.5 \times 10^{-9}}$	−0.55 (−0.74, −0.37)	$\mathbf{2.0 \times 10^{-7}}$
SM C24:0	0.05 (−0.07, 0.18)	0.45	0.07 (−0.07, 0.20)	0.39	0.19 (0.06, 0.31)	$\mathbf{1.9 \times 10^{-2}}$	0.21 (0.08, 0.34)	$\mathbf{1.2 \times 10^{-2}}$
H1	−0.26 (−0.40, −0.11)	$\mathbf{3.7 \times 10^{-3}}$	−0.25 (−0.40, −0.10)	$\mathbf{7.0 \times 10^{-3}}$	−0.20 (−0.36, −0.04)	0.62	−0.17 (−0.34, 0.01)	0.15

Using RBN, we found 10 significantly changed metabolites between night shift and day shift groups in both basic and full LMEM models. These 10 metabolites consisted of seven acylcarnitines, phenylalanine, PC ae C38:3 and SM C24:0 (Table 4).

We observed several consistent changes when comparing the three normalization methods. Acylcarnitine C10:2 was significantly decreased in night shift after applying any of the three normalization methods and the levels of nine metabolites were significantly altered in two out of three normalization methods (Figure 2B).

2.4. Metabolites Associated with Night Shift in the Chronotype—Stratified Analyses

We further investigated the influence of chronotypes on the concentration of 15 metabolites (identified with the creatinine normalization) in urine. We found that working night shifts significantly influenced 11, zero and four metabolites in nurses with early, intermediate and late chronotype, respectively (Table 3 and Table S2). In the early chronotype group, the 11 identified metabolites comprised 10 acylcarnitines (C5, C7-DC, C8, C10, C12, C14, C14:1, C14:1-OH, C14:2, C14:2-OH) and SM C24:0. In the late chronotype group, the levels of C10:2, C12, phenylalanine and SM C24:0 were changed when comparing night shift to day shift. Two metabolites, C12 and SM C24:0, were significantly altered both in early and late chronotype groups.

When comparing the results of combined and stratified analyses, 13 metabolites showed consistent changes in relation to night shift, whereas glycine and serine were only significant in the combined analysis (Table 3 and Table S2).

3. Discussion

Our main findings indicate that night shift work influences the metabolism of female nurses. The most changes concerning the metabolic profiles could be observed during night shift in nurses in the early chronotype class.

3.1. Identified Metabolites Largely Depend on the Applied Normalization Method

From a methodological point of view, our study is unique with respect to the application of three normalization methods to our multiple-time-point metabolomics data of nurses working in both night and day shifts.

Urine dilution can vary based upon water consumption and other physiological and pathophysiological factors, and, consequently, the concentrations of metabolites in urine also vary. Creatinine is a by-product of muscle metabolism. It is excreted from the body primarily through glomerular filtration. Creatinine is influenced by various factors, such as age, exercise but also physiological processes like the kidney tubule processing [19,22–24]. Creatinine -normalization is a commonly used approach, which makes comparison of results between studies feasible. To evaluate the total endogenous metabolic output in urine, osmolality can measured, which represents a direct measure thereof but can also be reduced by impaired kidney function [18,25,26].

Compared to creatinine, osmolality is usually not influenced by diurnal rhythms, diet, activity, age, stress or health state [18,27]. In our study, we observed a high correlation between measured creatinine and osmolality values, which is consistent with previous findings of r value of 0.75 [28]. However, regarding our used metabolite panel, the correlation coefficients between creatinine- and osmolality normalized values were low. We observed a low overlap of significant metabolites for creatinine and osmolality normalization, although we considered many influencing factors such as BMI and age in our statistical analysis.

We further present a new approach, RBN, which takes each metabolite's excretion kinetic into account, allowing for a dilution correction per metabolite. Comparison of these normalization methods showed that they are appropriate for different research questions since they showed consistent results only for one metabolite, acylcarnitine C10:2. Creatinine-based and RBN methods seem particularly suitable for acyl carnitines, phosphatidylcholines and sphingomyelins whereas osmolality-based

normalization seemed more suitable for amino acids. As to which normalization method to use depends on the study design and needs to be answered according to the research question. We focus on the creatinine normalization, as it has been used frequently and enables the comparison of our results with those of other studies.

3.2. Elevated Levels of Acylcarnitines May Result from Impaired Fatty Acid Oxidation

The increased urine levels of acylcarnitines during night shift could indicate an impairment of fatty acid oxidation. Acylcarnitines are imported and exported into/from the cell via the organic cation/carnitine transporter 2 (OCTN2) cell membrane carrier (Figure 2C) [29]. Due to the higher "social jet lag" and consequent sleep deprivation/restriction in nurses with an early chronotype, we hypothesize that these participants could be especially sensitive to night shift and show the most pronounced metabolic signs of sleep deprivation. Previous studies observed elevated blood levels of medium-chain acylcarnitines in healthy participants with acute sleep deprivation [9,10]. Due to an increased acylcarnitine concentration in blood, the renal OCTN2 carrier is saturated, yielding in a decreased renal reabsorption of acylcarnitines from urine [30]. We observed increased acylcarnitines concentration in urine during night shift, especially for nurses with early chronotype, which is in line with those previous reports. Furthermore, medium- and long-chain acylcarnitines produced in the kidney via fatty acid oxidation are directly secreted to the urine [31]. Inside mitochondria, the fatty acid oxidation is influenced by the NAD+ levels [8,12,15]. The nicotinamide phosphoribosyl transferase (NAMPT), a rate limiting enzyme of NAD+ synthesis, is regulated by the CLOCK/BMAL1 (Circadian Locomoter Output Cycles Kaput/Brain and Muscle Aryl hydrocarbon receptor nuclear translocator-Like 1) protein complex [32–34]. The transcription factor complex CLOCK/BMAL1 is a key modulator for the circadian rhythm [34]. The increased urine levels of acylcarnitines during night shift are likely to be due to a reduced circadian expression of NAMPT in shift working nurses (Figure 2C), leading to low NAD+ levels [32,33,35]. Moreover, acyl-CoA dehydrogenases (ACADs) catalyse the oxidation of long-chain fatty acids [36]. ACADs are subject to daily oscillations and prone to be influenced by shift work (Figure 2C) [36]. The metabolism of branched chain amino acids (BCAA) like leucine and isoleucine is catalysed via isovaleryl-CoA dehydrogenase, an enzyme from the ACAD family and might therefore be subject to perturbed circadian rhythms [37]. The observed medium chain acylcarnitine C14:2-OH is product of C14:2-OH-CoA which is metabolized by the bifunctional enzyme EHHADH (Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase) [31]. The expression of EHHADH is highly dependent on the circadian clock gene *Bmal1*. Increased levels of C14:2-OH could be a sign of impaired activity of EHHADH via an reduced expression of Bmal1 in the kidney [31,38,39]. Additionally, a downregulation of *EHHADH* was associated with an impaired BCAA catabolism [40].

3.3. Strengths and Limitations

Our study has several advantages. First, our study is based on urine samples. As the collection of urine is non-invasive and does not required a medically trained expert. However, this matrix is significantly less standardized than blood. Therefore, we applied three normalization methods. Second, we used waking-up urine samples, which is a proxy of fasting samples. As the spontaneous urine samples were collected in the course of a real-life study, we could not obtain urine after the recommended 8-hour-fasting period. Third, the comparison of the metabolites profiles of nurses working in night shift and day shift was based on the identical group of individuals (68 nurses working in both shifts). Fourth, to account for differences in sex steroids and glucocorticoids, which reflect the stress or menstrual phase, we designed the study by including a 4 week pause between day shift and night shift.

Our study is limited by several factors. First, the number of participants used in our study was small, particularly in the stratified analysis. This led to a reduced statistical power. In order to address the problem of potential under- or over fitting, we applied two different sets of confounders. Metabolites reported in this study required to be significant in both settings (basic and full models). Second, the interpretation of our results is based on the proceedings and metabolite pathways from

studies in cells or tissue models whereas the metabolites in our study were measured in urine that represents the last stage of metabolite degradation before its elimination. Moreover, metabolite concentrations may show tissue- and organ-specific regulation like kidney function or in muscle mass [23,24]. Third, we could not consider the potential influence of nutritional intake or of the duration of sleep to our multiple-time-point data. In our study, we tried to minimize this limitation by using waking-up samples. Fourth, we did not address potential diurnal changes which could influence the metabolite profiles. Reasons, therefore, were that many of our analysed samples were taken more than 12 h after the previous urine donation, which leads to an elimination of such diurnal effects. Fifth, our study was exclusively based on female participants.

3.4. Summary and Conclusions

Our results show an effect of night shift work on the metabolite values in urine. Out of 44 examined metabolites, 31 (about 70%) showed significantly altered concentrations by applying three normalization methods (creatinine-, osmolality- and regression-based normalization). We observed a low overlap of significant metabolites among the three methods. One metabolite was consistently identified with all three normalization methods and nine other metabolites (about 29%) were significantly altered in two out of three normalization methods. Individuals in the early chronotype class show the most significant metabolic changes. These were reflected by increased levels of acylcarnitines and altered concentrations of several amino acids in urine after night shifts. Independent studies, also including men, need to be conducted to confirm our finding that the individuals with intermediate or late chronotype classes show less affects in their metabolite profiles, when working in night shift.

4. Material and Methods

4.1. Study Design and Study Participants

For the current study, 100 female workers between 25 and 65 years of age were recruited at the clinical study site Bergmannsheil in Bochum, Germany. Exclusion criteria for study participation were (1) current pregnancy; (2) breastfeeding less than half a year ago; (3) past or present fertility medication; (4) prior cancer diagnosis.

After receiving a detailed explanation of the study protocol, each participant provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the ethic review committee of the medical faculty at the Ruhr University Bochum, Germany (No. 3840-10).

The recruited study participants (N = 100) were assigned to shift working (SW) and non-shift working (non-SW) categories. The SW group (N = 75) consisted of nurses working both, day shift and night shift. A pause of four weeks between day and night shift assessments was scheduled to account for potential hormonal changes over time (such as the menstrual cycle) (Figure 1A). The non-SW group (N = 25) consisted of women working only day shift. During day shift, nurses worked for up to four consecutive days (Monday–Thursday) (for both SW and non-SW) and up to five consecutive days (Monday–Friday) during night shift. The core working time for nurses of the day shift was scheduled from 6 a.m.–2 p.m. (SW) and 8 a.m.–4 p.m. (non-SW), while the night shift lasted from 9 p.m.–6 a.m. (SW) (Figure 1A). The group of non-SW nurses was excluded from the further analysis of metabolite profiles as their sociodemographic characteristics and particularly their diverse disease states would not allow a reliable analysis and comparison of the metabolite profiles.

Throughout day shift and night shift, urine samples and information on diet, sleep and medication were collected.

4.2. Chronotype Classification

Participants' chronotypes were calculated based on their sleep timing, as assessed by the Munich Chronotype Questionnaire for shift-workers ($MCTQ^{Shift}$), an adaptation of the standard MCTQ [21,41].

Specifically, mid-sleep (the time point midway between falling asleep and waking up) on work-free days is a proxy for circadian phase that can be derived from entries to the MCTQ and chronotype then is defined as mid-sleep corrected for sleep debt accumulated over the past work week [21,41–43]. The MCTQ^{Shift} allows calculating mid-sleep times for different work shifts (e.g., early, late, night) and chronotype then is usually derived from the mid-sleep on work-free days after late shifts [21]. In our study, 97 women provided information about their habitual sleep times in different shifts. Twenty participants reported that they used an alarm clock on all work-free days in all work shifts. For these 20 participants, we used mid-sleep on workdays with late-shifts to calculate their chronotype. For three participants, we calculated chronotype based on their sleep-debt corrected mid-sleep on work-free days after early shifts. Data of all 97 participants were then grouped into early, intermediate and late chronotypes based on thirds (33.3%) of the sorted mid-sleep time points [21]. Nurses with chronotype <3:37 a.m. were defined as early chronotypes (N = 32), nurses with chronotype between 3:37 a.m.–4:25 a.m. were defined as intermediate chronotypes (N = 32) and nurses with chronotype >4:25 a.m. were defined as late chronotypes (N = 33).

4.3. Urine Samples

All spontaneous urine samples throughout observation period were collected in 100 mL SARSTEDT disposable plastic containers, stored at 9 °C for a maximum of 24 h before being aliquoted to 1.5 mL Eppendorf tubes and deep frozen at −80 °C. Out of the collected 3640 urine samples, 2990 were measured for metabolite profiles including 2921 samples of the 97 nurses with chronotype information. Due to potential influences on the metabolite profile and on the sleep quality, individuals with diabetes (N = 4), vegetarians (N = 2) and women with extreme sleep apnoea (N = 1) were excluded from the SW group, resulting in 68 participants with 2278 urine metabolite profiles [8,30,44]. Of those, 424 were logged by study participants as waking-up urine samples, indicating the first urine donation after waking up (Figure 1B). On average, waking-up urine samples were donated at 05:17 (SD = 01:17) a.m. during day shift and at 12:52 (SD = 03:04) p.m. for night shift.

4.4. Targeted Metabolite Profiling

Each urine sample was measured with the Absolute*IDQ*^{TM} p150 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) using FIA-ESI-MS/MS (flow injection-electrospray ionisation-triple quadrupole mass spectrometry) [15,45]. In 10 µL urine, 162 metabolites were quantified (Table S1). We applied the same quality control (QC) criteria as in our previous study [15]. Overall, 2990 urine samples were measured in two batches. The QC was conducted separately for each metabolite and for each batch. In total, 44 metabolites passed the QC: free carnitine, 25 acylcarnitines (Cx:y), 13 proteinogenic amino acids, creatinine, hexoses (sum of hexoses), two phosphatidylcholine acyl-alkyl (PC ae) and one sphingolipid (SM C24:0) (Table S1). The abbreviations Cx:y depicts x number of carbons and y double bonds of all chains, respectively.

4.5. Osmolality Measurement

Measurements were performed using a Gonotec Osmomat 030 (Berlin, Germany). Freezing-point depression was used to determine osmolality [osm/kg] in waking-up urine samples.

4.6. Normalization Approaches

Urine samples where normalized to the respective creatinine and osmolality values, respectively [46,47]. Furthermore, a novel approach, regression based normalization (RBN), was applied [48]. In RBN, the dependency of urinary metabolite on the dilution variation is estimated from the data which allows for nonlinear functional relations. Nonlinear relations can occur in dynamically influenced urinary data.

4.7. Statistical Analysis

Normalized metabolite values were log-transformed and standardized (mean = 0 and standard deviation = 1). We used LMEM to compare the metabolic profiles between day shift (used as reference in the current study) and night shift of the SW group. A combined analysis, based on metabolite values of 68 participants, as well as a stratified analysis based on the respective chronotype class (early, intermediate and late) was conducted. For each metabolite, we calculated basic and full LMEMs. The basic model was adjusted for chronotype value and batch effect. To account for the sociodemographic differences between the study groups, the full model was additionally adjusted for BMI, age, smoking status, thyroid disease status, total years of shift work, respective day of shift block and time since last urination [12,49,50].

To account for multiple testing of the 44 used metabolites, false discovery rate (FDR, Benjamini Hochberg) was used as significance cut-off.

Statistical analyses were performed with SAS 9.4 (SAS Institute, Cary, NC, USA).

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-1989/8/3/45/s1, Figure S1. Correlation plot for age and chronotype value, Table S1. List of 162 quantified metabolites, Table S2. Results of 15 metabolites significantly altered by night shift work in combined and chronotype-stratified analysis based on creatinine-normalized values in the basic model.

Author Contributions: S.R. and R.W.-S. designed the study, M.R., K.B., E.B., J.H., B.R. and S.R. conducted the pre-analytical experiments and analysed the data; C.P. and J.A. (Jerzy Adamski) supervised and performed metabolomics measurements; S.B., M.C., M.T., J.A. (Jonathan Adam), M.H.d.A., H.J.G., H.D., T.K., V.H., T.I., D.P., T.B. (Thomas Behrens), T.B. (Thomas Brüning) and H.L. contributed to data acquisition, or to interpretation; M.R., S.B., M.C., M.T. and R.W.-S. wrote the paper. All authors critically reviewed the manuscript, revised the article and approved the final version of the submitted manuscript.

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