Four- and six-hour urinary albumin excretion is a valuable alternative to 24-h urinary albumin excretion in male db/db mice

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Abstract

In mouse (Mus musculus) models of diabetic nephropathy (DN), one of the most important read-outs is the 24-h urinary albumin excretion (UAE). The 24-h urine collection is usually performed by single housing mice in metabolic cages on wire mesh without enrichment. This is known to be stressful for the mice. Therefore, it was investigated if shorter urine collections would be sufficient to get reliable assessments of albuminuria. Twenty-one diabetic (C57BLKS-Lepr^db/db) and ten non-diabetic mice (C57BLKS-Leprd^b/+) were placed in metabolic cages at 15 and 20 weeks of age (WoA) for 24 h. Urine samples were taken at 4, 6, 18 and 24 h and albumin and creatinine concentration were measured. Four- and 6-h UAE was found to correlate significantly with 24-h UAE. Furthermore, a significant correlation was found between 24-h UAE and albumin:creatinine ratio (ACR) in the 4-h sample. However, the strength of the correlation between ACR and 24-h UAE was weaker than between the 4- and 24-h UAE. This suggests that normalising to creatinine may not provide additional value to the 4-h urine collection. In conclusion, the strong correlation between 4- and 6-h UAE and 24-h UAE indicates that the collection period can be considerably reduced. This refinement could reduce stress and increase welfare of the db/db model and potentially be applied to other DN models.

Keywords: albuminuria, animal welfare, db/db mice, diabetic nephropathy, metabolic cages, refinement

Introduction

Albuminuria is one of the key features of diabetic nephropathy (DN) and is used in diabetic patients to stage disease and predict progression of DN. This early manifestation of DN is caused by changes in the structure and function of the filtration barrier of the glomeruli as well as the tubular reabsorption which result in increased excretion of albumin in the urine (Gross *et al* 2005; Birn & Christensen 2006; Moresco *et al* 2013).

While animal models mimicking the more advanced changes in DN are still being developed, assessing the early changes in the kidney by measuring the urinary excretion of albumin is still a widely used read-out in these models (Breyer *et al* 2005; Brosius *et al* 2009; Azushima *et al* 2018). In mice (*Mus musculus*), albuminuria is usually measured by a total 24-h urinary albumin excretion (UAE) or albumin:creatinine ratio (ACR) in a spot urine sample. It has been shown previously that in some mouse models there is a poor correlation between ACR and UAE (Qi *et al* 2005). Since it is still not certain which of the two is the better read-out, many publications choose to report both which has also been recommended by the Animal Models of Diabetic

Complications Consortium (AMDCC) (Brosius *et al* 2009). One of the major disadvantages of using the 24-h UAE in DN models is the need for a full day of urine collection. This is usually done by housing the mice in metabolic cages, where they are single housed on wire mesh without enrichment or bedding; factors which are all known to induce stress in rodents (Manser *et al* 1995; Bartolomucci *et al* 2003; Hoppe *et al* 2009; Bangsgaard Bendtsen *et al* 2012; Kalliokoski *et al* 2013).

Furthermore, we have observed that the db/db mouse strain, which was used here, seems unable to maintain a high food intake in the metabolic cages, which is possibly due to mobility challenges or stress. This leads to weight loss and the mice often have difficulties in recovering from being metabolic cage-housed which may, in the worst case, lead to mortality or cause early euthanasia.

Moreover, consideration has been given to the possibility that mice may drink insufficiently while metabolic cage-housed, thereby becoming susceptible to dehydration. This may provide at least part of the explanation for the observed weight loss and slow recovery and perhaps also giving rise to variation in the urine read-outs.

Figure 1
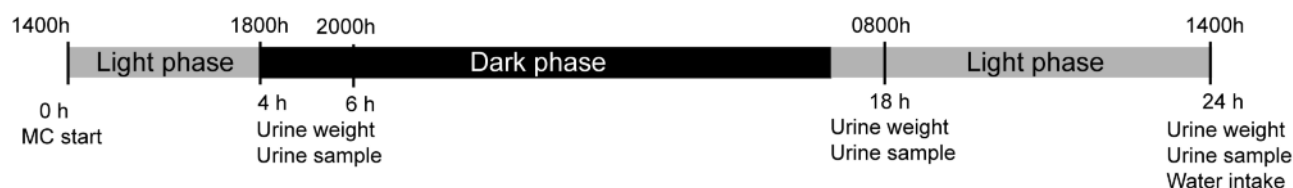
Timeline for metabolic cage housing and urine sampling.

Stress and weight loss during the 24-h urine collection impinge, considerably, on animal welfare within this and other DN models. It would therefore be highly desirable for mice to spend less time in metabolic cages. Shortening the collection period could refine this model and potentially also reduce the number of mice lost during these studies.

The main aim of this study was therefore to investigate if a shorter collection period may be sufficient for assessing albuminuria as an alternative to 24-h urine collection. Furthermore, the ACR was measured in the same urine samples in order to study how this read-out correlates with 24-h UAE and thereby investigate whether this could increase the usefulness of these shorter urine collections.

Materials and methods

Ethical statement

This animal experiment was carried out in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes and approved by The Animal Experiments Inspectorate, Ministry of Environment and Food of Denmark (license number 2014/15-0201-00429). Mice showing signs of compromised health (inadequate activity/moribund) or mice losing 20% bodyweight were euthanased.

Mice and housing

Study animals consisted of 21 male C57BLKS-Lepr[db/db] (db/db) and ten male C57BLKS-Lepr[db/+] (db/+) delivered from Charles River Laboratories (Calco, Italy) at eight weeks of age (WoA). The decision was taken to only use one sex since, in mice, diabetes has been found to be more severe in males. The number of db/+ and db/db mice included in the study were chosen based on experiences from previous studies.

Mice were housed in groups of 10–11 in type IV open cages (595 × 380 × 200 mm [length × width × height]) from Scanbur, Karlslunde, Denmark in cabinets (NOVOtainer, Scanbur, Karlslunde, Denmark). *Ad libitum* access to tap water (both from the watering system in the cabinet and extra water bottles in the cage lid) and to the chow diet NIH-31M was provided. Paper nesting material was supplied as enrichment along with wooden blocks, a cardboard tube (Brogaarden ApS, Lynge, Denmark) and a plastic hide (special order, Bach Ventilation ApS, Søborg, Denmark). Once daily the mice were given a portion of water-softened chow which was previously found to act as a beneficial supplement for diabetic mice (Nørgaard *et al* 2018). The

mice were kept at 25°C, 30–60% relative humidity and in a 12-h day/night cycle. Lights came on at 0600h and were switched off at 1800h. Microchip implants were used as a method of identification.

In order to monitor general well-being, mice were weighed at least twice weekly as well as before and after being housed in the metabolic cages. Furthermore, blood glucose was sampled once a week from the tail vein of non-fasted, conscious mice.

Experimental design

At 15 and 20 WoA the mice were housed in metabolic cages (Techniplast, Buguggiate, Italy) for 24 h from 1400h. At 4, 6 and 18 h the urine samples were weighed and 100 μL was taken out for albumin and creatinine measurements. The remainder of the urine was left in the tubes and at 24 h the total amount of urine was weighed and samples taken out for albumin and creatinine measurement (see Figure 1).

Water intake was measured in the metabolic cages by weighing the water bottles before and after the 24 h.

After being in the metabolic cages for the final time, mice were anaesthetised using isoflurane anaesthesia induced using an induction chamber, followed by euthanasia by intra-cardiac perfusion with saline.

Urine albumin and creatinine measurement

For albumin measurement, 10 μl urine was diluted 20× in 50 mM TBS, pH 8.0, 1% BSA, 0.05% Tween20 (E101, Bethyl Laboratories Inc, Montgomery, USA) and stored at –20°C until analysis. Albumin concentration was measured in all samples using an assay based on an ELISA kit from Bethyl Laboratories Inc (E90-134, Bethyl Laboratories Inc, Montgomery, USA) as described previously (Nørgaard *et al* 2018). Creatinine was analysed in the urine samples using HPLC-UV (Accela LC-System with Dionex Ultimate 3000 RS UV-detector, Thermo Scientific, San Jose, CA, USA).

Statistical analysis

Data were processed using GraphPad Prism software. To analyse the relationship between the albuminuria measurements in the shorter urine collections and the 24-h UAE, the data were analysed by linear regression analysis. The correlation between ACR and 24-h UAE, as well as between water intake and urine output, was analysed using Pearson's correlation. A *P*-value of 0.05 or less was considered statistically significant.

Figure 2
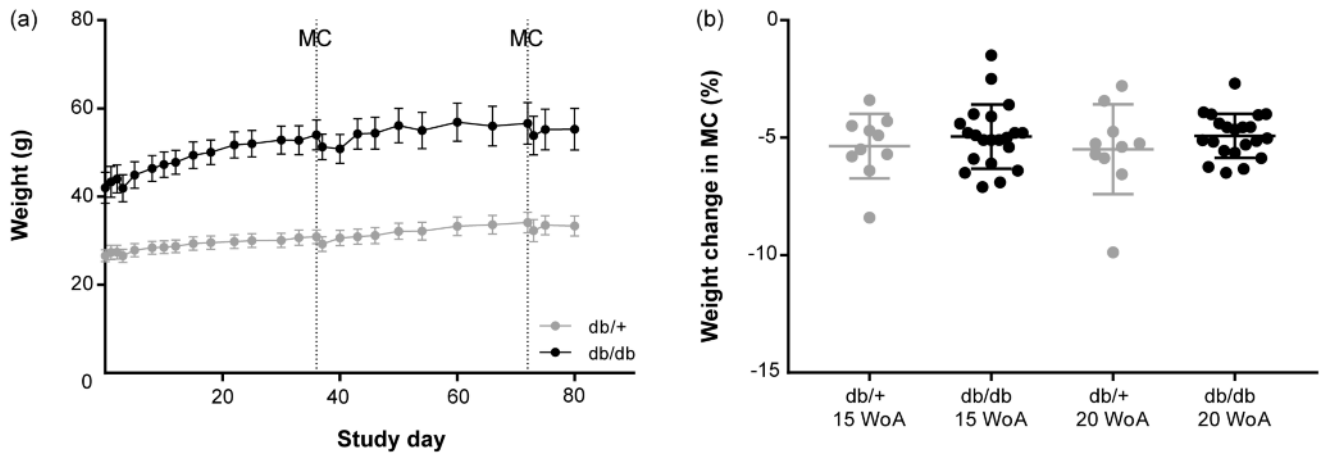
Mean (± SD) weight change shown during (a) the entire study, db/+ (n = 10), db/db (n = 21) and (b) the 24-h metabolic cage housing (MC), db/+ (n = 10), db/db (n = 21).

Figure 3
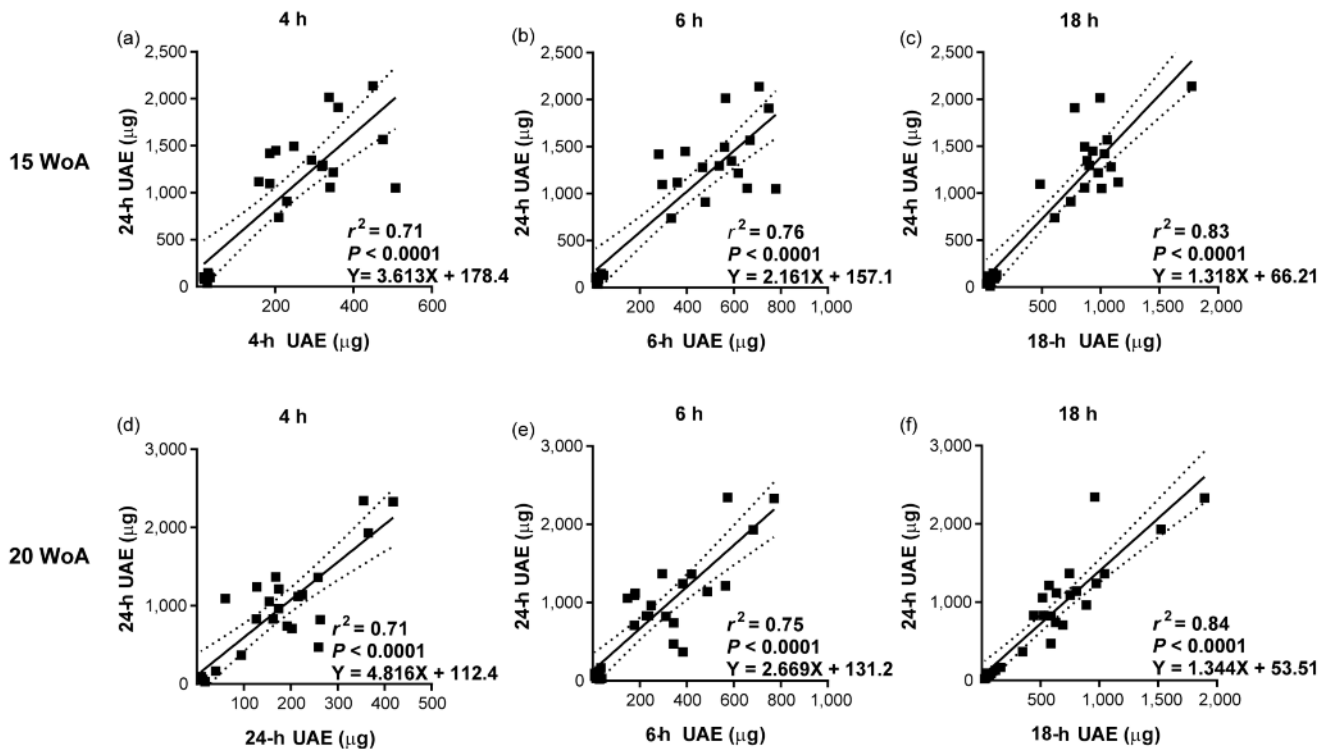
Linear regression analysis of relationships at 15 weeks of age between (a) 4- db/+ (n = 8), db/db (n = 17), (b) 6- db/+ (n = 8), db/db (n = 17) and (c) 18-h db/+ (n = 9), db/db (n = 17) samples UAE and 24-h UAE and at 20 weeks of age between (d) 4- db/+ (n = 5), db/db (n = 19), (e) 6- db/+ (n = 8), db/db (n = 19) and (f) 18-h db/+ (n = 9), db/db (n = 19) samples UAE and 24-h UAE. db/+ and db/db mice illustrated by grey and black dots, respectively.

Figure 4
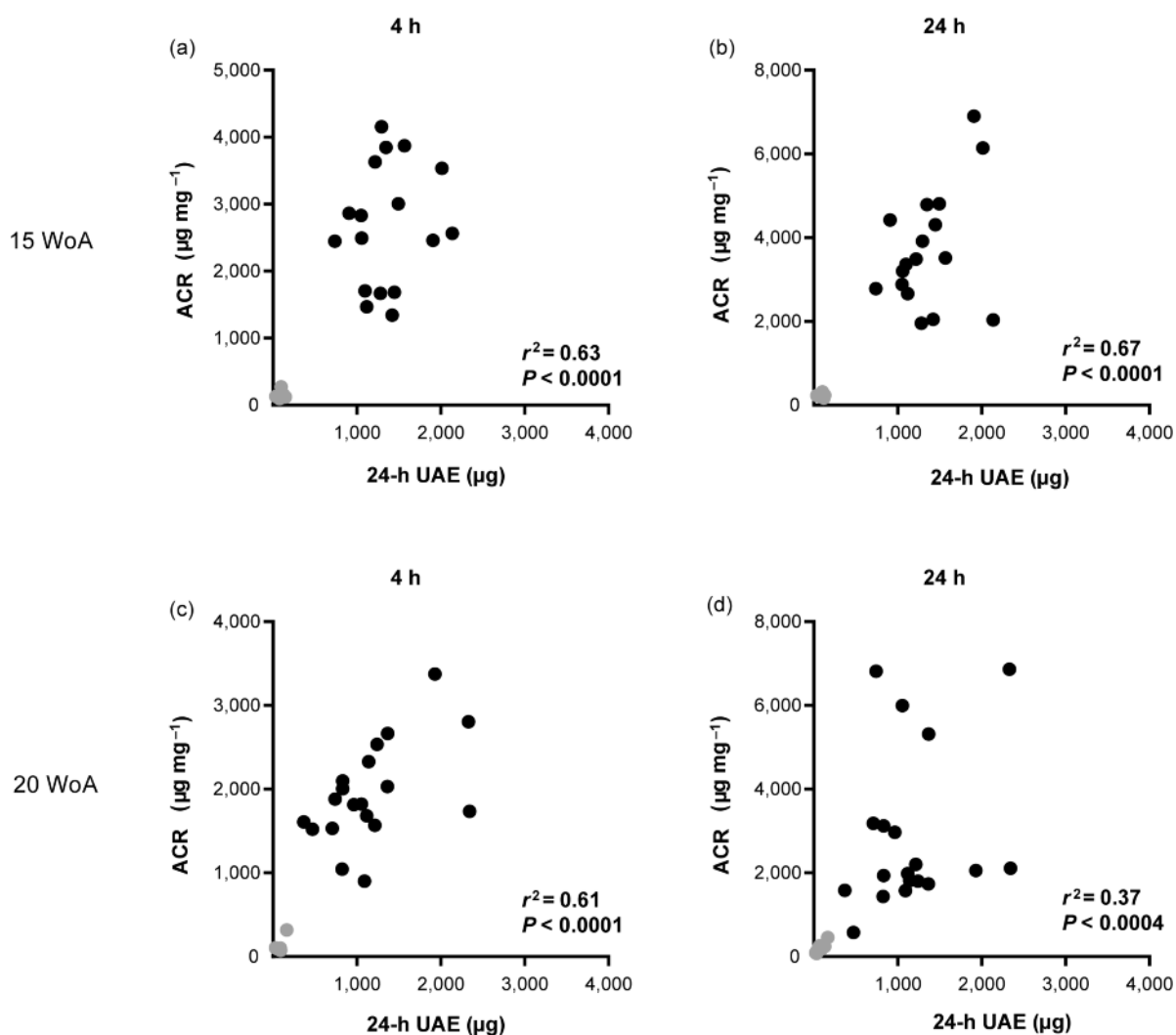
Relationship at 15 weeks of age between ACR and 24-h UAE for (a) 4- db/+ (n = 8), db/db (n = 17) and (b) 24-h sample db/+ (n = 7), db/db (n = 18) and at 20 weeks for (c) 4- db/+ (n = 5) db/db (n = 19) and (d) 24-h sample db/+ (n = 9), db/db (n = 19). db/+ and db/db mice illustrated by grey and black dots, respectively. Analysis via Pearson's correlation.

Results

Weight loss

The weight of the mice was measured at least twice weekly as well as before and after housing in the metabolic cages for 24 h. The weight curve for the entire study can be seen in Figure 2(a). In the first study week (at 10 WoA), a pilot study was run in which all the mice were placed in the metabolic cages for 24 h. This explains the drop in weight at this time-point. The data from this pilot study will not be included here. At 15 and 20 WoA (days 36 and 72), the mice were metabolic cage-housed for 24 h and at these time-points a drop in weight for both db/+ and db/db mice was observed. The weight change for the two collection periods in the metabolic cages can be seen in Figure 2(b). Apart from weight loss, no mortality or adverse events were observed.

Albumin excretion

As described in Figure 1, the mice were housed in metabolic cages for 24 h from 1400h and urine samples were collected at 4, 6, 18 and 24 h. In all samples, albumin was measured in order to assess the correlation between 4-, 6- and 18-h UAE and 24-h UAE. In Figure 3, data from all sampling time-points are shown from both 15 and 20 WoA. The albumin excretion in the shorter collection samples were found to correlate significantly with 24-h UAE.

In every data set some data-points are missing, due partly to an insufficient urine volume collection especially by db/+ mice and technical issues with the metabolic cages.

Albumin:creatinine ratio

Creatinine levels were additionally measured in the urine samples in order to investigate how well the ACR correlates with the 24-h UAE in the sample taken at 4 h (Figure 4[a]-[b]).

Figure 5
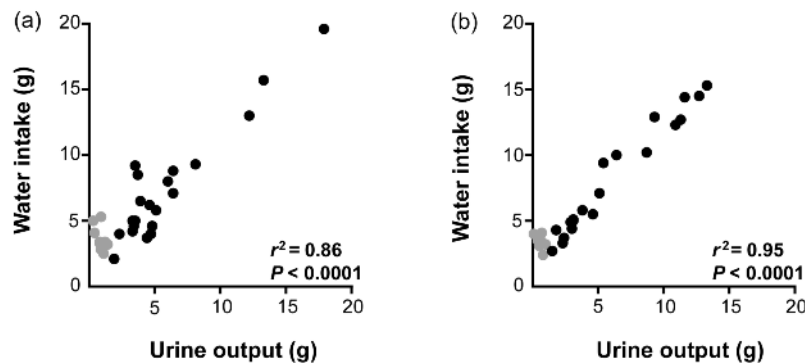
Relationship between water intake and urine output at (a) 15 weeks for age, db/+ (n = 9), db/db (n = 21) and (b) 20 weeks of age, db/+ (n = 10), db/db (n = 20). db/+ and db/db mice illustrated by grey and black dots, respectively. Analysis via Pearson's correlation.

This was done to assess if normalising to creatinine instead of volume improves the usefulness of these shorter collections in predicting the albuminuria. The ACR measured in the 4-h sample was found to correlate significantly with the 24-h UAE at both 15 and 20 WoA.

Creatinine was also measured and ACR calculated in the 24-h samples (Figure 4[c]-[d]). The ACR measured in the 24-h sample was likewise found to significantly correlate with the 24-h UAE at both 15 and 20 WoA; however, the strength of the correlation was found to be weaker at 20 WoA.

Water intake

Since the diabetic db/db mice often have a hard time recovering from being housed in metabolic cages, we wanted to assess their water intake during urine collection. This could offer an indication whether dehydration could be part of the explanation for the challenges during recovery.

As shown in Figure 5, water intake and urine output during the 24 h is found to be significantly correlated at both 15 and 20 WoA.

Discussion

With increasing prevalence of diabetes and DN being the most common cause of end-stage renal disease in humans, the use of animal models in the study of pathogenesis and screening of new therapies is essential. The search for new improved animal models is continuous but, additionally, the pursuit of welfare in DN studies must remain a priority since these are often prolonged due to the slow disease progression in most of these models.

Albuminuria is an important read-out in mouse models of DN and urine for this measurement is often collected over 24 h in metabolic cages. Multiple studies have shown housing in metabolic cages to be highly stressful for mice (Hoppe *et al* 2009; Kalliokoski *et al* 2013), findings reflected in our own results showing mice to suffer substantial weight loss during the collection period (Figure 2). We posit that the time mice need to spend in metabolic cages to reliably assess albuminuria could be reduced without detracting significantly from the overall accuracy of the read-out.

Previously, it has been observed that db/db mice struggle to recover from 24-h metabolic cage housing, with a high incidence of weight loss and constipation in the days and sometimes weeks to follow. It was, therefore, speculated whether this could be explained in part by insufficient water intake during housing in such a stressful environment. However, water intake and urine output indicate that mice take in sufficient amounts of water and that dehydration is unlikely to be the main reason for the long recovery (Figure 5). Food intake was not measured here, but it would be helpful to assess this parameter for mice within metabolic cages.

By taking urine samples from the metabolic cages at several time-points, we found that 4- and 6-h UAE correlated significantly with 24-h UAE both at 15 and 20 WoA (Figure 3). Our results suggest that a 4- or 6-h urine collection could be considered reliable to assess albuminuria in male db/db mice instead of the standard 24-h measurement.

In the same urine samples, creatinine concentration was additionally measured to investigate the relationship between the ACR and 24-h UAE. A significant correlation was found between ACR measured at 4- and 24-h UAE (Figure 4). However, the strength of the correlation was not increased by normalising to creatinine instead of volume which indicates that measuring ACR, instead of UAE, does not enhance the usefulness of the 4-h urine collection when assessing albuminuria in these mice.

However, reducing the collection period to 4 h could increase the risk of attaining insufficient urine from the non-diabetic mice, thereby detracting from the effectiveness of the research — a factor observed in this study. At 20 WoA we did not get any urine from 4 db/+ mice at 4 h. However, 2 h later only one mouse was still to urinate. This finding indicates that collecting for 6 h might decrease the risk of insufficient urine samples in non-diabetic mice. As expected, because of polyuria in the db/db mice, it was possible to collect urine samples from all diabetic mice within 4 h. Therefore, in studies where the non-diabetic mice are not used in the comparison of albuminuria, the risk of insufficient urine amount does not appear an issue.

Due to the stress the metabolic cage imposes on the mice it would be desirable to avoid this method of collection entirely. However, finding a reliable alternative method for obtaining uncontaminated urine samples remains a challenge. Most alternative collection methods use some degree of handling to get the mice to urinate which cannot therefore be used for quantitative measurements (Kurien *et al* 2004). When deploying other methods where there is no intervention, such as using hydrophobic sand, there is a risk of the mice drinking the urine; especially when working with diabetic mice. However, it is suggested that this method could be a viable alternative for collecting urine for shorter periods, eg 2 h, if the urine samples are collected immediately after deposition or every half hour thereby reducing the risk of the urine being consumed (Hoffman *et al* 2018). In the current study, we did not measure ACR in spot urine samples, so we are unable to assess the relationship between ACR in a spot urine sample and 24-h UAE in these mice. However, it has been shown previously that the 24-h UAE and ACR on spot urine correlates poorly in some strains (Qi *et al* 2005). Therefore, we believe that reducing the collection time in the metabolic cages could provide an optimal solution for attaining reliable samples and data while reducing the stress impact on the mice. It would however be interesting to investigate in a separate study, whether shortening of the collection period does actually reduce weight loss and stress in the mice.

For reasons of practicality, the urine in this study was collected from 1400h onwards. It has been shown previously that urine excretion varies throughout the day as a result of circadian rhythm (Cambar *et al* 1981), and that db/db mice have an altered circadian rhythm probably as a result of polydipsia (Grosbellet *et al* 2016). Therefore, if a shorter collection is to be used it might be necessary to assess if changing the time-point of the sampling periods has an effect on the correlation with the 24-h UAE. Here, the 4-h urine sample was collected just before the light was switched off at 1800h. This sample was therefore collected entirely during the light phase. So, if a sample is taken in the light phase from morning, it might not have a major effect on the correlation. The results shown in Figure 3 indicate how the shorter samples can be used to calculate predicted 24-h UAE data which can be useful to compare with historical data. However, changing the strain of mice, time of collection period, etc could potentially change this relationship and new data should therefore be generated.

Currently, the possibility of using shorter collections times for assessing albuminuria, as an alternative to 24-h urine collection, has only been assessed in db/db mice but could be investigated in other DN models since this could improve overall animal welfare in other models where the 24-h UAE is an important read-out.

Animal welfare implications and conclusion

In conclusion, this study finds a strong correlation between 4- and 6-h UAE and 24-h UAE. This suggests that it is not necessary to have the mice housed in metabolic cages for 24 h to get a reliable measurement of albumin-

uria. It has been shown multiple times that metabolic cage housing is highly stressful for mice (Hoppe *et al* 2009; Kalliokoski *et al* 2013), a trend that continued here with us noting a marked drop in weight in the both db/+ and db/db mice. And, as albuminuria measurement is a highly important read-out in all DN models, it is crucial to refine the procedure of urine collection. It is our belief that shortening the collection period would be a substantial refinement of the procedure. In the current study, levels of stress hormone were not measured. However, even lacking data for stress levels, if 4 h of urine collection is sufficient to get a reliable UAE measurement in diabetic mice, we would recommend making it standard protocol for albuminuria assessment in mice. This major shortening of the urine collection period, could thereby considerably refine this and potentially other models of DN.

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