Distinct osmoregulatory responses to sodium loading in patients with altered glycosaminoglycan structure: a randomized cross-over trial

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Abstract

Background: By binding to negatively charged polysaccharides called glycosaminoglycans, sodium can be stored in the body—particularly in the skin—without concurrent water retention. Concordantly, individuals with changed glycosaminoglycan structure (e.g. type 1 diabetes (DM1) and hereditary multiple exostosis (HME) patients) may have altered sodium and water homeostasis.

Methods: We investigated responses to acute (30-min infusion) and chronic (1-week diet) sodium loading in 8 DM1 patients and 7 HME patients in comparison to 12 healthy controls. Blood samples, urine samples, and skin biopsies were taken to investigate glycosaminoglycan sulfation patterns and both systemic and cellular osmoregulatory responses.

Results: Hypertonic sodium infusion increased plasma sodium in all groups, but more in DM1 patients than in controls. High sodium diet increased expression of nuclear factor of activated t-cells 5 (NFAT5)—a transcription factor responsive to changes in osmolarity—and moderately sulfated heparan sulfate in skin of healthy controls. In HME patients, skin dermatan sulfate, rather than heparan sulfate, increased in response to high sodium diet, while in DM1 patients, no changes were observed.

Conclusion: DM1 and HME patients show distinct osmoregulatory responses to sodium loading when comparing to controls with indications for reduced sodium storage capacity in DM1 patients, suggesting that intact glycosaminoglycan biosynthesis is important in sodium and water homeostasis.

Trial registration These trials were registered with the Netherlands trial register with registration numbers: NTR4095 (https://www.trialregister.nl/trial/3933 at 2013-07-29) and NTR4788 (https://www.trialregister.nl/trial/4645 at 2014-09-12).

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Background
Disturbances in osmoregulation result in abnormal plasma sodium concentrations leading to either hypo- or hypernatremia; the most common electrolyte disorders in clinical practice [1]. Dysnatremia is to be found in one-third of elderly patients admitted to the emergency room and one-third of critically ill patients admitted to the intensive care unit [2, 3]. Dysnatremias have a large impact on both short- and long-term outcomes, as illustrated by many studies that demonstrate an association between dysnatremia and increased morbidity and mortality [2, 4–6]. Yet, the pathophysiology of disturbed osmoregulation remains poorly understood and complete understanding of water and sodium homeostasis is thought to be necessary for the improvement of outcomes [7].

In recent years, the classical view on sodium homeostasis has drastically changed by the revelation that sodium can be transiently stored within various tissues of the body in concentrations that far exceed those in plasma, including skin, muscle, and possibly the blood vessel wall, varying with dietary intake [8–10]. This adds a third dynamic compartment to the classical two-compartment model, which solely assumes equal osmolality in the intra- and extracellular space. Recently, the potential sodium buffering capacity in healthy subjects was estimated to be ~ 50 mmol (equaling ~ 3 g of salt (NaCl)) following acute hypertonic infusion [11]. Sodium storage is believed to be facilitated by negatively charged polymeric disaccharides called glycosaminoglycans (GAGs) [12, 13]. GAGs can be variously sulfated, and the sulfation degree may primarily determine the quantity of GAG-mediated tissue sodium accumulation [14–16]. Several studies in animal models and in healthy humans have shown that increased skin sodium storage (induced by high sodium intake) coincides with increases in GAGs and enzymes involved in the modification and degradation of GAGs [10, 14–18]. Also, nuclear factor of activated T-cells 5 (NFAT5; also called tonicity-responsive enhancer binding protein (TonEBP)), a transcription factor that is crucial for tissue specific cellular responses to hypertonic stress, responds to high tissue sodium concentrations [10, 14–18]. Thus, individuals with changes in GAG biosynthesis and/or structure may have altered sodium homeostasis and respond differently to sodium overloads. Whether patients with disturbed GAG metabolism are characterized by disturbed osmoregulatory responses to sodium is unknown.

We studied the osmoregulatory responses to acute and chronic sodium loading in type 1 diabetes mellitus (DM1) patients, known for acquired GAG remodeling, and patients with hereditary multiple exostosis (HME) with genetic alterations in GAG synthesis, and compared them to matched healthy controls. We assessed systemic osmoregulatory responses by measurements of plasma and urine osmolarity, and local cellular osmoregulatory responses by assessing NFAT5 (along with associated GAG changes) in skin biopsies. In DM1, a decrease in GAG synthesis and/or increase in GAG destruction results in decreased GAG content and GAG sulfation degree in a variety of organs and structures, including the kidney, skin and endothelial surface layer (luminal coating of blood vessel walls) [19–22]. Patients with HME, a rare autosomal dominant disorder with mutations in EXT-1 and/or EXT-2 genes that are responsible for polymerization of heparan sulfate (specific type of GAG), have a genetic form of GAG dysfunction [23]. Data from recent animal experiments using congenic EXT-1EXT-2 mice show a damaged endothelial surface layer and lower skin sodium-to-glycosaminoglycan ratio [24, 25].

Methods
Study design
We carried out two similar prospective randomized cross-over intervention studies in DM1 patients, HME patients, and healthy controls. We included male, non-smoking, normotensive (Blood pressure (BP) < 140/90 mmHg) subjects between 18 and 40 years old with a body mass index (BMI) < 30 kg/m². For inclusion, DM1 patients had to have normal and stable renal function (creatinine clearance > 60 ml/min and < 6 ml/ min decline per year) and HbA1c (42–86 mmol/mol) during 6 months preceding the study. Use of renin-angiotensin system blocking agents was allowed for DM1 patients, but these were discontinued prior to the study visits using an interval that was equal to or exceeded 5 times the elimination half-life. All subjects pursued an 8-day low sodium diet (LSD; < 1.2 g (50 mmol) sodium/day) and high sodium diet (HSD > 4.8 g (200 mmol) sodium/day) in a randomized order, with 1–2 weeks between diets. Diet order was determined by block randomization via sealed envelopes by the study investigators. Diet status was not masked for study subjects or investigators during the dietary intervention. Diets were pursued with the help of a dietary list, based on

Keywords: Sodium, Glycosaminoglycan, Osmoregulation, Type 1 diabetes, Hereditary multiple exostoses, Nuclear factor of activated T-cells 5
which participants could compile their own diet. This list advised to resemble the normal diet of the individual as much as possible—e.g., by adding extra salt instead of changing the whole dietary pattern (aiming to keep other nutrients as stable as possible). We checked dietary compliance by collecting 24-h urine samples on day 3, 6 and 8. After each diet, skin biopsies were obtained.

**Acute sodium loading experiment**

On day 8 of LSD, an additional 1-day acute sodium loading experiment was performed in all subjects. After standardized low-sodium breakfast and lunch, subjects were asked to fully empty their bladder, and hypertonic sodium was administered intravenously in 30 min. In each subject, the total amount of infused sodium equaled 5 mmol/L of total body water (estimated to be 60% of total body weight). By adding a 20% NaCl solution (range 29–57 mL in DM1, 35–55 mL in HME, and 29–50 mL in healthy controls (HC)) to 500 mL of 0.9% NaCl, the infused volume was only slightly different. After infusion, we obtained blood and urine samples at timed intervals during a 4-h period. During this period, water intake was standardized to a total of 350 mL divided in similar portions on similar time points in all subjects. DM1 patients took their standard dose of insulin during the day. The results of the acute sodium loading experiment in healthy controls and data on urinary GAGs were previously published [11, 26].

**Calculations acute sodium loading experiment**

To assess whether there were indications for (differences in) sodium buffering after acute sodium loading in DM1 patients and HME patients, we compared observed plasma and urinary cation levels with expected plasma and urinary cation levels according to the Adrogue-Madias and the Nguyen-Kurtz formulas (Additional file 1: Appendix 1), as described previously [11, 27]. In short, with these formulas, we calculated expected plasma sodium levels based on the administered sodium and water quantity, taking into account total body water and initial plasma sodium [28, 29]. Furthermore, we calculated how much cations should be present in urine to account for plasma sodium decreases that took place after initial increases. The discrepancy between observed and expected values indicates which fraction of infused sodium—provided that plasma potassium concentrations remain stable—is stored in the body. In DM1 patients, plasma sodium levels were corrected for glucose levels, using a correction factor of 1.6 mmol/L per glucose increase of 5.5 mmol/L [30].

**GAG analysis in 24-h urine collection**

Glycosaminoglycans were enzymatically digested into disaccharides, and results were reported as previously described [31, 32]. Disaccharides D0a4 and D0a10 were formed after enzymatic digestion with chondroitinase B representing dermatan sulfate disaccharides, and with chondroitinase C representing chondroitin sulfate disaccharides. Disaccharide concentrations were adjusted for creatinine concentration of the urine samples. A validated high performance liquid chromatography with mass spectrometry/mass spectrometry method was used to quantify urinary excretion of heparan sulfate, dermatan sulfate, and chondroitin sulfate disaccharides. Values below the lower limit of quantification were assigned as of the numeric half value of the lower limit of quantification.

**Other laboratory analyses**

Blood was collected in 4.5-ml lithium heparin tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for analysis of plasma sodium, potassium, osmolality, and creatinine. Three-milliliter tubes with 5.4-mg spray-dried K2 ethylenediaminetetraacetic acid (BD Vacutainer) were used for hematocrit determination. All blood samples were centrifuged at 2000 g for 10 min at 18 °C and analyzed within 60 min of collection. We used the indirect ion selective electrode method to measure plasma sodium and potassium and urine sodium and potassium. Plasma and urinary osmolality were determined by freezing point depression.

**Immunohistochemistry**

To assess cellular osmoregulation responses to sodium, sections of formalin-fixed and paraffin-embedded skin biopsies were investigated for presence of NFAT5 and glycosaminoglycans by histochemistry and immunostaining as described in detail in Additional file 1: Appendix 2. Briefly, sections were incubated with rabbit IgG anti-NFAT5 (ThermoFisher), followed by AlexaFluor488-conjugated goat IgG anti-rabbit IgG (Jackson ImmunoResearch). NFAT5-stained sections were counterstained using Hoechst 33,342 nuclear stain (ThermoFisher). For detection of total GAGs, tissue sections were stained with Alcian Blue 8GX (Sigma-Aldrich, Saint Louis, MO, USA) at pH 2.7 and at pH 1.0, at which strongly acidic sulfated glycosaminoglycans are stained more selectively. Sections were counterstained with Nuclear Fast Red (Sigma-Aldrich, Saint Louis, MO, USA). For staining of dermatan sulfate GAGs, VSV-tagged phage display derived antibody LKN1 (Radboudumc, Nijmegen, the Netherlands) was applied to detect 4/2,4-di-O-sulfated dermatan sulfate domain [33] and...
GD3A12 antibody (Radboudumc, Nijmegen, the Netherlands) to detect IdoA-Gal-NAc4S dermatan sulfate domain [33, 34]. For heparan sulfate GAGs, VSV-tagged phage display derived antibodies (HS4C3, AO4B08 and HS4E4) (Radboudumc, Nijmegen, the Netherlands) were applied as primary probes to detect specific sulfation motifs in heparan sulfate chains. In particular, HS4C3 binds 3-O-sulfated heparan sulfate chains with preference for the fully sulfated IdoA250GlcNS3S6S domains [35], AO4B08 binds N-sulfated, 2-O-sulfated, and 6-O-sulfated heparan sulfate chains, corresponding to the IdoA25-GlcNS6S domain which lacks 3-O sulfations [36] and HS4E4 binds heparan sulfate domains containing both N-sulfation and N-acetylation and in general heparan sulfate chains with low sulfation grades since the presence of 6-O-sulfated sites inhibits binding of HS4E4 [36]. Regarding the spectrum of these three domains of heparan sulfate, we therefore consider binding of HS4C3 to represent a highly sulfated heparan sulfate domain, AO4B08 a moderately sulfated heparan sulfate domain and HS4E4 a low sulfated heparan sulfate domain. Bound antibodies were detected using subsequently rabbit IgG anti-VSV (Sigma), poly-AP conjugated goat IgG anti-rabbit IgG (Bright Vision, Immunologic) and PermaBlue/AP substrate (Diagnostic Biosystems). The sections were counterstained with Fluorescin Ulex Europaeus Agglutinin (Vector Laboratories) which was detected by Ulex Europaeus Lectin Type I Rabbit anti Ulex (Dako) and BrightVision Poly-HRP anti-rabbit IgG (Immunologic). HRP activity was visualized using NovaRED (Vector Laboratories).

Image analysis

Epifluorescence imaging of NFAT5-stained sections was performed (Leica DM5500B, Wetzlar, Germany) with an HCX PL APO 63×/1.40–0.60 oil immersion objective and applying filters A4 for Hoechst and L5 for AlexaFluor488 fluorochromes. From each section six to ten images were recorded of the epidermis and conjointly of the dermis using a DFC365 FX camera and LASX software (Leica). For manually selected regions of interest, background was subtracted using the ‘rolling ball’ plugin with ImageJ2 software [37]. The number of nuclei was analyzed for each image. Next, the fractional AlexaFluor488-stained surface area (representing NFAT5) was measured and expressed per nucleus for at least 100 nuclei per region of interest. Brighfeld microscopy was applied for sections stained with Alcian Blue, LKN1, GD3A12, HS4C3, AO4B08 and HS4E4 using either a BX51 with UPlanXAPO 20×/0.80 objective and DP70 camera (Olympus, Tokyo, Japan) or a slide scanner (IntelliSite Ultra Fast Scanner; Philips, Eindhoven, the Netherlands) with UPlanXAPO 40×/0.95 objective (Olympus). In recorded images either dermis or epidermis were selected as region of interest. Subsequently, the ‘color deconvolution’ plugin with setting for FastBlue/FastRed/DAB was applied to isolate the Alcian Blue and FastRed or the PermaBlue and NovaRed signals [38]. The thresholds were set with the AutoThreshold function “Moments”. Finally, the fractional stained area of the regions of interest was measured for PermaBlue stained sections.

Furthermore, to validate the semi-quantitative measurements and to specify the measurements on the cellular level, images from all stainings were analyzed qualitatively by two blinded reviewers, according to a pre-determined scoring system. The extent of staining was scored for the dermal extracellular papillary matrix, papillary cells and endothelium. For the diet type, the researchers remained blinded during all staining and analysis processes.

Statistics

Continuous data are expressed as mean and standard error of the mean (SEM). Multiple imputation was used to estimate missing values, and potential outliers were detected using Grubb’s test. Paired t-test or Wilcoxon test was used to detect differences between LSD and HSD, depending on the data distribution. To determine differences between patient groups an unpaired t-test or Kolmogorov–Smirnov was performed, as appropriate. Test hypotheses were (i) \( H_0: \) DM1 patients ≠ healthy controls, \( H_A: \) DM1 patients = healthy controls and (ii) \( H_0: \) HME patients ≠ healthy controls, \( H_A: \) HME patients = healthy controls. To test correlations between variables Spearman’s correlation coefficient was used. For the acute hypertonic saline infusion experiment, repeated measurements two-way ANOVA with Tukey post-hoc test was used to account for repeated time points. The statistical analysis was executed using SPSS software (version 24.0, IBM Analytics, Armonk, NY, USA). Figures were acquired using GraphPad Prism (version 7.0d, GraphPad, La Jolla, CA, USA). A P-value of <0.05 was considered significant.

Results

Population and dietary intervention

Between September 2013 and November 2015 we screened 9 DM1 patients, 8 HME patients, and 19 healthy controls. One of the DM1 patients was excluded after screening because of a BMI > 30 kg/m² and one HME patient withdrew informed consent after screening due to inability to adhere to the protocol instructions. Of the healthy controls, four subjects withdrew their consent after inclusion before randomization and three subjects were excluded before randomization (one due to high...
BP and two others due to difficulties with blood drawing). There was no loss-to-follow-up and all randomized subjects were included in our analyses (Fig. 1). Baseline characteristics are depicted in Table 1.

**Table 1 Baseline characteristics of study subjects, determined at screening before commencement of the diets**

|                      | Type 1 diabetes patients (n = 8) | HME patients (n = 7) | Healthy controls (n = 11) |
|----------------------|----------------------------------|----------------------|--------------------------|
| Age (year)           | 28.1 (2.0)*                      | 26.6 (3.2)           | 22.7 (1.2)               |
| BMI (kg/m²)          | 22.8 (0.9)                       | 24.4 (1.1)           | 22.0 (0.6)               |
| Total body weight (kg)| 77.4 (3.3)                      | 78.8 (2.8)           | 75.7 (2.0)               |
| Hematocrit (L/L)     | 0.45 (0.01)*                     | 0.44 (0.01)          | 0.43 (0.01)              |
| Glucose (mmol/L)     | 8.1 (1.5)*                       | 4.7 (0.2)            | 5.1 (0.2)                |
| Plasma sodium (mmol/L)| 139.0 (0.5)                     | 140.1 (0.3)          | 140.0 (0.5)              |
| Plasma potassium (mmol/L)| 4.4 (0.1)               | 4.0 (0.1)            | 4.2 (0.1)                |
| Plasma osmolality (mOsm/kg)| 295 (2)                    | 290 (1)              | 287 (5)                  |
| Plasma creatinine (μmol/L)| 72 (3)*                       | 73 (2)*              | 81 (3)                   |
| Urine sodium (mmol/24 h)| 193 (28)                      | 154 (24)             | 166 (20)                 |
| Urine potassium (mmol/24 h)| 74 (14)                       | 72 (4)               | 71 (5)                   |
| Supine systolic blood pressure (mmHg)| 124 (2)            | 118 (2)              | 121 (3)                  |
| Supine diastolic blood pressure (mmHg)| 64 (3)                | 63 (2)               | 59 (2)                   |
| Supine heart rate (bpm)| 56 (2)                         | 64 (4)               | 61 (2)                   |

Data are depicted as mean (SEM). Data were tested using an unpaired t-test compared to controls. *P < 0.05 vs. healthy controls.
All subjects adequately followed the LSD and HSD, as assessed by 24-h urine sodium excretion. Urinary sodium excretion was comparable among groups, equaling a mean of ~1.5 g estimated NaCl intake per day for LSD and a mean of ~19 g estimated NaCl intake per day for HSD (Table 2). Potassium intake was successfully kept stable (Table 2).

**Urinary GAGs at baseline**

On day 8 of LSD, urinary GAGs were measured to assess overall GAG status. DM1 patients had less urinary dermatan sulfate compared to healthy controls. Although urinary levels of heparan sulfate were similar for DM1 patients and healthy controls, the extent of sulfation of heparan sulfate was diminished in DM1 patients (Fig. 2). HME patients had lower along with less-sulfated urinary heparan sulfate than healthy controls, but, in contrast, had more urinary dermatan sulfate (Fig. 2). In both DM1 patients and HME patients, the di-sulfated dermatan sulfate disaccharide D0α10 was detected, contrasting the results found in healthy controls, where only mono-sulfated dermatan sulfate disaccharide was present.

**Acute sodium loading experiment**

To assess osmoregulation, plasma sodium changes subsequent to acute hypertonic saline infusion were measured (Fig. 3). Five minutes succeeding infusion, there was a plasma sodium increase compared to baseline in all three groups, after which plasma sodium decreased again, ending ~2 mmol above baseline 4 h after infusion. Plasma sodium changes in DM1 patients differed significantly compared to healthy controls (P < 0.05) with higher mean increases in DM1 patients up to 1 h after infusion (Fig. 3a). Additionally, in DM1 patients, the initial 5-min rise of plasma sodium was higher than expected according to the Edelman-based Adrogue-Madias and Nguyen-Kurtz formulas, whereas in HME patients and healthy controls no discrepancies were found. (Fig. 3b). Changes in other laboratory parameters are depicted in Additional file 1: Figure S1 and Table S1. The amount of cations assumed to be in urine to account for plasma sodium decreases taking place between 5 min, 2 h and 4 h after infusion, was calculated using the Adrogue-Madias and Nguyen-Kurtz formulas. These amounts were substantially higher than the amounts truly observed in all groups (Fig. 3c and Additional file 1: Figure S2). This difference between observed and expected values represents the fraction of infused sodium and potassium ions that could not be traced back to plasma or urine (i.e., "cation gap"; and in case of stable plasma potassium most likely representing sodium ions i.e., "sodium gap") and did not significantly differ compared to controls.

**Chronic sodium loading experiment**

Skin NFAT5 and the presence of glycosaminoglycans were analyzed with immunohistochemistry in all groups. In healthy controls, HSD increased the extent of dermal NFAT5 expression (Fig. 4, Table 3). In other groups, no differences in dermal NFAT5 expression could be observed between both diets. The location of NFAT5 in endothelial cells shifted from a predominantly cytoplasmic pattern in LSD to a both cytoplasmic and nuclear pattern in HSD in healthy controls (Fig. 4b–f). No such HSD-induced nuclear translocation of NFAT5 could be observed in other groups. Alcian Blue staining at pH 2.7, representing total skin GAG, was similar in the papillary matrix in all groups after LSD (Table 3) (Additional file 1: Figure S3 and S4). There were salt-induced fluctuations in highly sulfated residues (after staining at pH 1.0) in healthy controls and HME patients. However,
these fluctuations were not present in patients with DM1, showing a stable extent of highly sulfated residues upon HSD (Table 3) (Additional file 1: Figure S3). An increase or similar presence of total GAG content was present in DM1 patients, whereas in healthy controls a decrease was observed in the majority (Table 3) Additional file 1: Figure S4).

In the skin of HME patients, we semi-quantitatively measured an increase of the IdoA-Gal-Nac4S domain (GD3A12) in the epidermis after HSD (Fig. 5a, b). With qualitative analysis, we were unable to observe a cell-specific increase. Epidermal presence of the 4/2,4-di-O-sulfated (LKN1) domain of dermatan sulfate was not affected by HSD (Fig. 5c, d). In the dermis, we were unable to observe clear salt-induced differences in dermal expression of both domains of dermatan sulfate in all groups.

For dermal heparan sulfate, the presence of IdoA2S-GlcNS6S as detected by AO4B08, used as marker for moderately sulfated heparan sulfate, increased after HSD in the dermis of healthy controls (Fig. 6). We observed a strong increase of AO4B08 staining in both the extent of cellular staining in papillary dermis and the endothelium upon HSD (Fig. 6d) (Additional file 1: Figure S5). In DM1 and HME, the presence of the AO4B08 heparan sulfate domain showed no specific salt-induced alterations in the
dermis. The extent of staining for HS4E4 (used as marker for relatively low sulfated heparan sulfate domains) and HS4C3 (IdoA2S-GlcNS3S6S, used as marker for highly sulfated heparan sulfate domains) in the dermis showed no clear sodium-induced alterations in either of the groups. We semi-quantitatively measured a higher percentage of positively stained area of HS4E4 in the dermis of DM1, however this could not be confirmed qualitatively (Additional file 1: Figure S6).

With our semi-quantitative analysis, we observed HSD-induced changes in the dermis of healthy controls, where both NFAT5 and the AO4B08 domain of heparan sulfate (moderately sulfated heparan sulfate domains) content increased per surface area. For both, this mainly resulted from an increased staining pattern in endothelial cells. Semi-quantitative results of NFAT5 and moderately sulfated heparan sulfate domains showed a positive correlation during LSD (n = 12, r = 0.76, p = 0.004) (Fig. 7).

To assess the association between the observed skin cellular response to HSD and body osmoregulation in healthy controls, we tested correlations between dermal NFAT5 expression and plasma and urine variables affected by HSD (Table 2). This showed that in healthy controls, NFAT5 expression was negatively correlated with plasma osmolality during HSD, while in DM1 and HME patients this correlation was absent (Fig. 8).

Discussion
We investigated whether patients with either acquired or genetically determined GAG disturbances exhibit altered sodium and water homeostasis. We demonstrated that in response to hypertonicity, healthy volunteers showed an increase in both NFAT5 expression as well as in the abundance of the AO4B08 epitope of heparan sulfate (used as marker for moderately sulfated heparan sulfate domains), while these changes were absent in DM1 and HME subjects. Whilst in healthy volunteers skin GAG changes were present in the dermis, an increase in epidermal dermatan sulfate was observed in HME subjects. The absence of cellular osmoregulatory responses to chronic salt loading in DM1 subjects might reflect a reduced capacity for tissue sodium loading which can
explain the unexpectedly high increase in plasma sodium concentration following hypertonic saline infusion.

The finding that HSD increases NFAT5 expression and influences the sulfation degree of heparan sulfates in the dermis of healthy controls extends previous observations in healthy volunteers, in which a 1-week HSD increases skin sodium, macrophages, vascular endothelial growth factor-C (VEGF-C), and lymphatics [18, 39]. The increase of the osmotically sensitive transcription factor NFAT5 reflects electrolyte accumulation in excess of water, resulting in local hypertonicity as shown by animal studies of the group of Titze et al. [10, 14, 15, 17, 40]. Moreover, in other studies of this group, HSD led to increases in skin sodium, macrophage influx, and lymphatic expansion via expression of VEGF-C in macrophages [10, 14, 15, 17, 40]. These changes coincided with increases in GAG content and increases in specific GAG chain elongation enzymes, which are crucial for dermatan sulfate and chondroitin sulfate synthesis [14]. A cross-sectional study with human abdominal tissue obtained during surgery showed close correlations with sodium content and GAG content [16]. While these data, together with in vitro experiments showing GAGs are able to bind sodium, indicate that GAGs may have a key role in tissue sodium accumulation, and lymphatic expansion via expression of VEGF-C in macrophages [10, 14, 15, 17, 40]. These changes coincided with increases in GAG content and increases in specific GAG chain elongation enzymes, which are crucial for dermatan sulfate and chondroitin sulfate synthesis [14]. A cross-sectional study with human abdominal tissue obtained during surgery showed close correlations with sodium content and GAG content [16]. While these data, together with in vitro experiments showing GAGs are able to bind sodium, indicate that GAGs may have a key role in tissue sodium accumulation, and lymphatic expansion via expression of VEGF-C in macrophages [10, 14, 15, 17, 40]. These changes coincided with increases in GAG content and increases in specific GAG chain elongation enzymes, which are crucial for dermatan sulfate and chondroitin sulfate synthesis [14].

We observed HSD-induced NFAT5 and GAG changes in the dermis of healthy volunteers, whereas epidermal changes in this group were not as clearly apparent. We observed increased extent of nuclear NFAT5 in endothelial cells, implicating increased activation of NFAT5 target genes. In the dermis, endothelial cells are the first cells undergoing increased osmotic stress during increased intravascular sodium concentrations. In this context, it is remarkable that DM1 and HME did not show cellular responses to osmotic stress during HSD. In a previous study showing increased skin sodium content after HSD, the exact localization of skin sodium accumulation failed to be determined [18]. Also, specialized 23Na-MRI, which visualizes sodium accumulation in the skin, was not able to distinguish sodium accumulation in the epidermis from dermal sodium accumulation [41]. Our observations indicate a role for the dermis in skin sodium buffering during HSD. However, detection of accumulation of skin sodium ions at high spatial resolution is required to substantiate these findings further.

In DM1 patients, no HSD-associated skin changes in NFAT5 or GAGs could be observed. This is in line with the absence of HSD-induced skin macrophage and lymphatic changes in these patients (in contrast to healthy subjects, who showed macrophage increases and lymphatic expansion in response to HSD) [39]. We previously hypothesized that this possibly reflects the absence of sodium influx, for example as a consequence of an already saturated buffer due to high skin sodium content at baseline. In the present study, higher urine (low sulfated) heparan sulfate excretion were found at baseline in DM1 compared to controls. Furthermore, DM1 patients showed no HSD-induced fluctuations in highly sulfated skin GAG expression (Alcian Blue staining), while this was observed in healthy controls. Although this may fit with higher sodium content in this patient group, as has been demonstrated earlier in type 2 diabetes patients, this is yet to be established for type 1 diabetes [42, 43]. Also, our acute sodium loading experiment suggests reduced sodium storage capacity in DM1 patients, as plasma sodium increase was higher in this patient group compared to healthy controls and higher than expected according to the Edelman-based formulas. Edelman et al. empirically showed that plasma water sodium = 1.11 * (total exchangeable body sodium + potassium)/total body water—25.6 [44]. Since our experimental set-up ensured that changes induced by hypertonic saline infusion in both total exchangeable body sodium + potassium cations and total body water were similar in the three groups, this would either mean altered slope (1.11) or altered y-intercept (−25.6) in DM1 patients when

Fig. 4 Expression of NFAT5 in the skin. a Semi-quantitative analysis. b, d Upon LSD no specific differences in NFAT5 expression could be observed in the endothelium. c, e Paired observations showed that in healthy controls the location of NFAT5 in endothelial cells shifted from a predominantly cytoplasmic pattern in LSD to a both cytoplasmic and nuclear pattern in HSD. No such shifts could be observed in other groups. f Paired histological images (of which a selection containing the endothelium is shown) show that in healthy controls the expression of NFAT5 (green) in endothelial cells shifts from a predominantly cytoplasmic pattern in LSD (nuclei visualized by Hoechst staining (blue)) to a cytoplasmic plus nuclear pattern in HSD. In DM1 and HME no shift could be observed. NFAT5, nuclear factor of activated T-cells 5, LSD, low sodium diet. HSD, high sodium diet. DM1, type 1 diabetes. HME, hereditary multiple exostosis. HC, healthy controls. n = 8 (DM1), n = 7 (HME), n = 12 (HC). *P < 0.05. Data are presented as individual data with mean (SEM). Data were tested with a paired t-test or Wilcoxon test, as appropriate, to compare two diets, and with an unpaired t-test or Kolmogorov–Smirnov test, as appropriate, to compare two patient groups.
adoption of the classical two-compartment view of sodium and water homeostasis. The two factors that determine the slope of the Edelman experiments—i.e., the Gibbs-Donnan equilibrium and osmotic coefficient of sodium—are not different in DM1 patients, nor are the first two of the three factors that determine the y-intercept—i.e., respectively, plasma potassium, osmotically active non-sodium and non-potassium osmoles (such as glucose and urea), and non-osmotically stored sodium and potassium [45]. Therefore, both increased plasma sodium and the lack of HSD-associated skin changes in DM1 patients may reflect reduced skin sodium storage capacity. It is to be noted that we previously showed that there were no considerable differences in plasma renin and aldosterone responses between DM1 patients and controls, thereby ruling these factors out as potential underlying causes for the other observed differences [46].

Considering the increase of epidermis dermatan sulfate in response to chronic sodium load, one might speculate that HME patients seem to compensate in this way for their disturbance in heparan sulfate. Additionally, urinary dermatan sulfate in HME patients is higher than in controls. This might represent a compensatory mechanism consistent with the idea that reduction of one type of GAG could affect the production of the other, like the finding that somatic cells bearing mutations in EXT1 show increased chondroitin sulfate synthesis [47] and the observation that EXT1±EXT2± mice (mouse model for HME) show increased dermatan sulfate content compared to wildtypes [25]. Furthermore, in the urine of DM1 patients and HME patients the dermatan sulfate disaccharide D0a10 as present. D0a10 is a disaccharide that is ordinarily absent in healthy individuals, but is known to be found in mucopolysaccharidosis patients, who are characterized by deficiency or malfunctioning of lysosomal enzymes for GAG breakdown [48]. It remains unclear whether this structural alteration has effect on sodium binding capacity. In DM1, increased expression of heparanase and possibly sulfatases may be involved in modulation of HS, as has been documented for diabetic nephropathy [49, 50]. The present acute sodium loading experiment indicates that acute sodium

| Table 3 Overall results of paired observations of qualitative analysis |
|-------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                         | NFAT5           | Alcian Blue     | Alcian Blue     | Low sulfated    | High sulfated   | Low sulfated    | High sulfated   | Moderate          | High sulfated   |
|                         | pH1.0           | pH 2.7          | DS              | DS              | HS              | DS              | HS              | sulfated HS      | HS              |
| (GD3A12)                | (LKN1)         | (HS4E4)         | (AO4B08)        | (HS4C3)         |                 |                 |                 |                  |                 |
| Dermis endothelium      |                 |                 |                 |                 |                 |                 |                 |                  |                 |
| DM1                     | 0               | 0               | ↑               | ~               | ~               | ~               | ↓↓              | ~                | 0               |
| HME                     | 0               | ~               | ~               | 0               | ~               | ~               | ~               | ~                | ~               |
| HC                      | ↑↑              | ~               | ~               | ↑               | ~               | ↑               | ~               | ↑↑               | ~               |
| Dermis matrix           |                 |                 |                 |                 |                 |                 |                 |                  |                 |
| DM1                     | ↓               | 0               | ↑               | ~               | ~               | ↓               | ~               | ↓                | ~               |
| HME                     | ~               | ~               | ~               | ~               | ~               | ↓               | ~               | ~                | ↑               |
| HC                      | ~               | ~               | ~               | 0               | ~               | ~               | ↑↑              | ~                | 0               |
| Epidermis               |                 |                 |                 |                 |                 |                 |                 |                  |                 |
| DM1                     | 0               |                 |                 |                 |                 |                 |                 | ↑                | ↓↓              |
| HME                     | ↓               |                 |                 | 0               | ~               | ~               | ~               | ↑                | ~               |
| HC                      | ↓               |                 |                 | ~               | ~               | ↓               | ~               | ~                | ↑               |

Trends of changes in staining extent are shown as ↑↑ (strong increase), ↑ (increase), ↓ (decrease), ↓↓ (strong decrease), 0 (same extent of staining), ~ non-specific fluctuations. Alcian Blue staining of the epidermis was not scored, due to minimal differences in extent of staining that could not be visually assessed. DM1, type 1 diabetes. HC, healthy controls. HME, hereditary multiple exostosis.
buffering capacity in HME patients might not be different compared to controls, despite their disturbance in heparan sulfate polymerization, potentially explained by compensatory dermatan sulfate. As experimental studies showed that skin sodium accumulation was paralleled by elevated GAG content, one might expect that HSD-induced increase in skin dermatan sulfate coincides with increased skin sodium content [15]. However, we were not able to observe a significant increase in dermis dermatan sulfate and NFAT5 (as a reflection of increased skin sodium/osmolarity) in this patient group, possibly due to a power problem.

We are—to our knowledge—the first to translate existing preclinical hypotheses on the link between sodium and GAGs by studying osmoregulation responses after sodium loading in patients with GAG alterations. The indications for a link between GAG disturbances and altered osmoregulation responses motivate further research to confirm causality, as this will have significant clinical impact on the pathophysiology and management of dysnatremias and its associated adverse outcomes in clinical practice [2]. However, certain limitations deserve consideration. First, skin sodium content could not be directly assessed in this study, due to the limited amount of skin material that we could obtain from participants. We did, however, use NFAT5 expression as reflection of osmolarity, because of its known responsiveness to osmolarity increases (hence its alternative name ‘tonicity-responsive enhancer binding protein’) and known associations of NFAT5 loci with plasma osmolality on population level [51]. Also, interpretation of GAG measurements can be complex. Although we are the first to provide a closer look into specific types and specific sulfation grades of skin GAGs in the context of salt loading, it is impossible to perform stainings for every existing sulfation grade. Therefore, the presently studied sulfation grades of GAGs are not all-encompassing. Finally, although this study brings translation of the link between
GAGs and sodium one step closer, the co-occurrence of GAG alterations and indications for altered sodium homeostasis does not prove a causal relationship. For this, we still rely on studies that assess sodium homeostasis after intervening with GAG status. Sulodexide is an example of a GAG intervention since its main component is synthetic heparan sulfate. This drug was previously shown to restore the endothelial surface layer [52] and to lower blood pressure with borderline significant effects on plasma sodium [53]. Although these prospective data may point towards increased sodium buffering capacity, definitive proof is still lacking and assessment of sodium homeostasis/sodium buffering capacity after GAG interventions is warranted.
Conclusions

Two patient groups with GAG alterations—DM1 and HME patients—show altered osmoregulation responses to a sodium load, with indications for reduced sodium capacity in DM1 patients. Future research with interventions targeting GAGs is necessary to establish whether this relation is causal, which will significantly impact osmoregulation disorders and their management.

Supplementary Information

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Abbreviations

BP: Blood pressure; DM1: Type 1 diabetes; GAGs: Glycosaminoglycans; HC: Healthy controls; HME: Hereditary multiple exostosis; LSD: Low sodium diet; NFAT5: Nuclear factor of activated t-cells 5; TonEBP: Tonicity-responsive enhancer binding protein; VEGF-C: Vascular endothelial growth factor-C.

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Authors’ contributions

LV conceptualized the clinical trial. ROE and NR performed the acute sodium experiments and conducted the chronic sodium study. EW and SB analyzed the data from the acute sodium experiments. AQ en TK developed the used antibodies for the staining of glycosaminoglycans. EW, JO and JA designed and performed the skin stainings and analyzed the results. EW and JO wrote the manuscript. All authors reviewed the manuscript. LV and BB supervised the entire process. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The studies were conducted at the Academic Medical Center (Amsterdam, the Netherlands) after approval of the Academic Medical Center ethics committee. All subjects provided written informed consent. The trials were conducted in accordance with the Declaration of Helsinki and according to the original protocol (www.trialregister.nl; NTR4095, NTR4788), reporting adhered to CONSORT guidelines [55].

Consent for publication

Not applicable.

Competing interests

All authors have read the journal’s policy on disclosure of potential conflicts of interest. There are no competing interests to declare.

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