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Early life regional and temporal variation in filaggrin-derived natural moisturising factor, filaggrin processing enzyme activity, corneocyte phenotypes and plasmin activity: Implications for atopic dermatitis

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Abstract

Background

Filaggrin is central to the pathogenesis of atopic dermatitis (AD). The cheeks are a common initiation site of infantile AD. Regional and temporal expression of levels of filaggrin degradation products (natural moisturising factors, NMF), activities of filaggrin processing enzymes (bleomycin hydrolase; BH and calpain-1; C-1) and plasmin, and corneocyte envelope (CE) maturity in early life are largely unknown.

Objectives

We did a cross-sectional, observational study investigating regional and age dependent variations in NMF levels, activity of proteases and CE maturity in stratum corneum (SC) from infants to determine if these could explain the observed predilection sites for AD in early life.

Methods

We measured NMF, using a tape stripping method, at 7 sites in the SC of 129 children (<12 to 72 months) and in 3 sites in 56 neonates and infants (<48 hrs to 3 months). In

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37 of these neonates and infants corneocyte size, maturity, BH, C-1 and plasmin activities were determined.

Results

NMF levels are low at birth and increase with age. Cheek SC, compared with elbow flexure and nasal tip, has the lowest NMF in the first year of life and is the slowest to reach stable levels. Cheek corneocytes remain immature. Plasmin, BH and C-1 activities are all elevated by 1 month of age in exposed cheek skin, but not in elbow skin.

Conclusions

Regional and temporal differences in NMF levels, CE maturity and protease activities may explain the predilection for AD to affect the cheeks initially and are supportive of this site as key for allergen priming in early childhood. These observations will help design early intervention and treatment strategies in AD.

What's already known about this topic?

- Atopic dermatitis (AD) frequently starts in early infancy and the first eczematous lesions emerge on the cheeks.
- Filaggrin is a major structural protein in the stratum corneum (SC).
- Filaggrin deficiency is associated with the development of AD and, in the context of AD, food allergies and asthma.
- Filaggrin is metabolised into natural moisturising factors (NMFs) that can be measured in the SC.

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What does this study add?

- Regional differences in NMF levels, CE immaturity and protease activities may help explain why infantile AD most often initially affects the cheeks.
- Cheek skin may be highly relevant for allergen priming.
- Emollient therapy at the vulnerable cheek site might help prevent AD and/or food sensitization.

What is the translational message?

- Filaggrin processing, corneocyte maturity, and protease activities show regional and temporal differences in infant skin.
- These findings may explain disease patterns in early life AD

Key Words

Stratum corneum, atopic dermatitis, epidermal barrier, allergen sensitization, cheeks, transepidermal water loss, natural moisturising factor, proteases, corneocyte maturity

Abbreviations

AA: Total amino acids without their derivatives

AD: Atopic dermatitis

BH: Bleomycin hydrolase

C-1: Calpain-1

CE: Corneocyte envelope

FAA: Total free amino acids with their derivatives, PCA, UCA, citrulline and ornithine

FLG: Human filaggrin gene

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His: Histidine

HPLC: High-performance liquid chromatography

NMF: Natural moisturising factor

PCA: Pyrrolidone carboxylic acid

SC: Stratum corneum

SG: Stratum granulosum

TEWL: Transepidermal water loss

totNMF: His + PCA + UCA

UCA: Urocanic acid

UV: Ultraviolet

Introduction

Atopic dermatitis (AD) is the most common chronic inflammatory disease of childhood in the developed world.[1] Clinical manifestations of AD vary with age. In infancy, the first lesions most commonly emerge on the cheeks and the scalp.[2] A Danish study of 411 infants reported that AD lesions started on the scalp, forehead, ear, neck and cheek in infants, with the cheek being the most commonly involved region. [3] While infantile AD typically involves the facial skin, the nasal tip is lesion-free: the 'Yamamoto sign'.[4]

These well-recognised predilection sites for AD suggest distinct regional differences in skin structure and function.

Structural differences between infant and adult skin have been reported. [7, 8] Infant corneocytes have a smaller size compared with adults, correlating with a higher epidermal cell turnover rate compared to adults. [9] In contrast to adults, newborn babies do not show variation in corneocyte size between non-acral skin regions. Their

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corneocytes are uniformly small, similar in size to those of adult forehead.[10] This suggests consistently high epidermal turnover over the entire skin surface in neonates. Adaptive changes, both in SC water handling properties and skin surface pH occur in early life. In full-term neonates, transepidermal water loss (TEWL) data suggest a competent epidermal barrier function shortly after birth.[7, 11]

A defective skin barrier is a key feature of AD. [12] Filaggrin is a major structural protein in the SC and its constituent amino acids play a critical role in SC acidification and hydration. Filaggrin deficiency is associated with impaired barrier function and the development of AD[13] Filaggrin deficiency in children seems to define a specific endotype of AD, characterised by a predilection to exposed areas of the body, in particular the cheeks and hands.[14] Profilaggrin, the phosphorylated precursor to filaggrin, is expressed in the upper stratum granulosum (SG), where processing is initiated. A complex cascade of dephosphorylation and proteolysis liberates functionally active filaggrin monomers that, within the SC, are further cleaved into short peptides and finally degraded fully to form a cytoplasmic pool of hygroscopic amino acids and their derivatives, known as natural moisturising factor (NMF). Following deimination of arginine residues, the cysteine/aspartic proteases caspase-14, bleomycin hydrolase (BH) and calpain-1 (C-1), located mainly in the upper SC, have been shown to be important in enzymatic degradation of filaggrin. [15, 16] C-1 is also involved in the processing of profilaggrin to filaggrin, as well as in corneocyte maturation. [17, 18]

Natural moisturising factor modulates the pH of the SC surface; retention of water within the corneocytes and may exert antimicrobial activity. [1] Important amino acid derivatives include *trans*-urocanic acid (t-UCA) and pyrrolidone carboxylic acid (PCA).

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[1] The enzyme histidase converts histidine to t-UCA.[19] Glutamic acid is converted to PCA, by non-enzymatic degradation (cyclization) as well as enzymatic processes and overall, constitutes 12% of NMF.[20-22] In addition, non-filaggrin derived compounds such as lactate, urea, sugars and ions contribute to NMF. The amino acids and their derivatives that constitute NMF can be measured and closely correlate with filaggrin content. [23] The epidermis expresses several proteases that are involved in a range of key epidermal responses, including proliferation, differentiation, lipid barrier homeostasis and tissue remodeling. [24] Plasmin plays an important, but still not yet completely defined, role in the corneocyte maturation process and is a marker for SC stress and inflammation, even in areas lacking clinically observable inflammation. [25]

Why AD has a predilection for certain body sites and why the affected regions vary with age in childhood remain unknown. Age-related and regional differences in skin structure and function are likely important. Here, we investigated the regional and temporal expression of filaggrin-derived NMF together with filaggrin processing enzyme activities (BH & C-1), corneocyte phenotypes and plasmin activities in a large cohort of healthy neonates, infants and children in locations relevant for AD.

Subjects and Methods

Study population

In phase I of the study, healthy infants and children were recruited from March to September 2013. These infants and children were booked for elective surgical procedures in Our Lady's Children's Hospital, Dublin. Children were recruited if they did not have history suggestive of AD, or any other inflammatory skin disease. An experienced paediatric dermatologist (MAMcA) examined all infants. Other exclusion

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criteria from the study included subjects who had pyrexial illness in the preceding 2 weeks, those who had received immunosuppressive systemic therapy, such as oral corticosteroids, in the preceding 3 months, and patients whose ancestry was not exclusively Irish (4/4 grandparents). Non-Irish children were excluded to allow for accurate *FLG* mutation stratification. Seven body sites were tape stripped in phase 1: cheek, nasal tip, nape of neck, volar forearm at the elbow flexure, dorsal proximal mid upper limb, dorsal hand, and the buttock.

Phase II of the study was carried out in The National Maternity Hospital, Holles Street, Dublin (July -September 2014). Infants were recruited, both in the postnatal wards in the 48 hours following delivery, and in the early weeks of life in outpatient/ambulatory clinics, when attending for elective baby checks. Identical inclusion and exclusion criteria applied for phase II as for phase I. Three body sites were tape stripped in this cohort: cheek, nasal tip, and volar forearm at the elbow flexure. The number of body sites sampled had to be reduced in view of the very young age of these subjects. The 3 body sites in this cohort were selected because preliminary data from phase I of the study suggested that these body sites were most likely to be most informative. As this was an exploratory study, a definitive sample size that would allow statistically significant findings was difficult to predict. After Phase I yielded significant results, we calculated that the smaller sample size would be informative in phase II. The power analysis was based on the difference in NMF values obtained between skin sites C, T and E. The highest sample size of n=38 (power 80%, p=0.05, paired t-test) was obtained for the difference between C and E (mean 0.21 and SD 0.47). We included more children (n=59) as we expected that tape stripping might fail in neonates and infants.

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The study was conducted in accordance with the Helsinki Declarations and was approved by the Research Ethics Committee of Our Lady's Children's Hospital, Dublin, Ireland. Full written informed consent was obtained from all subject's parents.

Sampling of the stratum corneum by tape stripping

All sampled sites were emollient free for 24 hours before tape stripping. The SC was sampled using the previously described method. [23] Circular adhesive tape strips (3.8 cm^2, D-Squame, Monaderm, Monaco, France) were attached to the skin and, in all regions apart from the nasal tip, pressed for 10 seconds with a constant pressure (225 g/cm^2) using a D-Squame pressure instrument D500 (CuDerm, Dallas, TX, USA). On the nasal tip, the circular adhesive tape strip was cut in half, placed on the skin, and pressure was applied manually for 10 seconds.[26] Eight consecutive tape strips were sampled, all from the same site. Tape strips were stored at -80°C until analysis.

***FLG* genotyping**

All patients were screened for the 9 most common filaggrin (*FLG*) mutations found in the Irish population (R501X, Y2092X, 2282del4, R2447X, S3247X, R3419X, 3702X, S1040X, and G1139X) from DNA extracted from a blood sample or buccal swab. The methods used have been previously described.[27] All subjects that were heterozygous or homozygous for loss-of-function mutations in the *FLG* gene were excluded from the analysis.

Determination of filaggrin breakdown products in the SC

NMF component analysis (His, PCA, trans-UCA and cis-UCA) and total SC protein were performed on the 4th consecutive tape stripping according to the method described in

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details elsewhere.[23] These four components were combined to give a 'total NMF' value. NMF components in the SC from each tape stripping were extracted with 500 µL 25% (*w/w*) ammonia solution. After evaporation of the ammonia extract, the residue was reconstituted in 250 µL pure water and analyzed by HPLC-UV analysis. Due to incomplete extraction recovery of SC proteins by ammonia, the second extraction of proteins from the tape stripping was performed with 0.1 M KOH solution during 24 hrs. The proteins were determined in both extracts by using Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, USA). The levels of NMF components in the SC were normalized for proteins and expressed as mmol NMF/g protein.

NMF depth profiling

To ensure that NMF measurement in different age groups and sites were not affected by varying SC thickness, NMF profiling was done on consecutive tape strips in 13 subjects. NMF was assessed in the 2nd, 6th and 8th tape stripping in subjects from <48 hours old to 3 months of age at the cheek and elbow sites.

Stratum corneum maturation assays

The first tape stripping from 37 subjects at 2 skin sites, the cheek and elbow were investigated for CE maturity. This was done using differential Nile red and immunostaining for the late epidermal differentiation marker involucrin, using a modification of the previously described method.[28]

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Measurement of stratum corneum protease activities: calpain-1 and bleomycin hydrolase and plasmin

The same 37 subjects were assessed for plasmin, C-1 and BH activities. Protease activities were determined on the 5th tape stripping using previously reported methods. [25, 28-31].

Statistical analysis

All calculations were performed by using Prism 6 software (GraphPad, San Diego, CA, USA). The distribution of data was tested by Shapiro-Wilk normality test. The applied statistical test is indicated within the figure or in-figure captions.

Results

Demographic data of subjects

One hundred and eighty-eight infants and children were recruited, 112 male and 76 female (Table 1). The average age, age range, and age groups are outlined in Tables 1 and 2. The demographics of the 37 subjects that had corneocyte maturity assays as well as C-1, BH and plasmin measured are outlined in supplemental table S1. *FLG* mutation status was determined in all subjects in phase I. In phase II we were unable to definitively establish the *FLG* status in 12 subjects because of poor DNA quality obtained from this subset of buccal swabs (supplemental table S2). We excluded these from analysis.

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Total NMF is low after birth, increases rapidly in post-natal life and is regionally determined, with the cheek skin being slow to mature.

In the first 48 hours after birth, the total NMF levels were low in all sampled regions. There was an increase in the total NMF levels in the early weeks of life in all regions. The nasal tip and the elbow flexure had a rapid increase in total NMF levels in the first 4 weeks of life, after which NMF levels off, with no further increase (Figure 1). In contrast, in cheek skin there was still a significant increase ($P<0.001$, Mann Whitney 2-sided paired test) in NMF between time periods of 1-11 months and 12-35 months. Furthermore, the rate of increase in levels of NMF in cheek SC was much slower (see Figure S1), and there was a significant ($r^2=0.28$; $P<0.0001$) linear response of NMF vs. age from 1 up to 72 months (Supplemental Figure S1). Of note, there was no variation in NMF levels with SC depth after 48h of age (Supplemental Figure S2). NMF levels on the remaining body sites sampled in phase I (nape of neck, dorsal hand, dorsal upper limb, and buttock) were stable over the ages of 1 month to 72 months of age. (Supplemental Figure S3). In the first year of life, the NMF values in the check SC were significantly lower as compared to the elbow (median of difference 0.15; $P <0.0001$) (Wilcoxon paired t-test), while this difference was not significant at ages >12 months (Supplemental Figure S4).

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The histidine/UCA ratio and the trans-UCA/cis-UCA ratio change rapidly postnatally and transition to a steady state more quickly in exposed skin sites compared to non-exposed sites.

The His to UCA ratio rapidly increases in the first 3 months of life in exposed sites, and reaches a steady state from 4 months of age onwards (approximately 2-3 fold). In the non-exposed elbow flexure skin the His to UCA ratio remains low for the first 3 months of life (Figure 2). The cis-UCA/totUCA ratio increases rapidly in postnatal life in exposed sites. The ratio of cis-UCA to the total amount of UCA (cis + trans isomer) in the cheek and nasal tip skin reaches the levels 30%- 50% around 1st year of life while the ratio between cis-UCA/tot UCA in the elbow flexure SC remains low (Figure3).

Bleomycin hydrolase and calpain-1 activities increase after one month of life and are higher in the cheek than the elbow.

The late stage filaggrin processing enzymes, BH and C-1 activities are similar in the cheek and elbow skin after birth. After 1 month of age the levels increase significantly in the exposed cheek skin and after 1 month of age, but not at the elbow (Figures 4 & S5).

Plasmin activity increases at one month of age in the cheek and correlates with trans-UCA/cis-UCA ratio in cheek skin

Plasmin activities are higher in the cheek compared with the elbow at birth (Figure 4). At one month, the plasmin activity generally increases in the exposed cheek skin. Plasmin activities correlate with trans-UCA/cis-UCA ratios at the cheek (Figure 5).

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Cheek corneocytes have a more immature phenotype, in contrast with elbow corneocytes.

The cheek corneocytes have an immature phenotype from birth, which does not change with age. This contrasts with the elbow corneocytes, which have a mature phenotype from birth (Figure 4). In keeping with an immature phenotype, the cheek corneocytes had higher cohesion – specifically, there was less SC protein removed so that the corneocytes were more tightly cohesive (Figure 6).

Discussion

Our results show that NMF is low at birth at all investigated skin sites and increases significantly over the first month of life, suggesting that the SC rapidly adapts to the dramatic environmental shift from *in utero* to *ex utero*. Our findings are in keeping with a previous study that measured free amino acids in the chest or back skin of 30 neonates and infants. [32] Similarly, Chittock *et al* demonstrated low NMF in the skin of 115 neonates at birth, with a significant increase in NMF by 4 weeks of age.[33] Low NMF in infant skin at birth is in keeping with reports in variations between neonatal and adult rat skin. Scott and Harding reported that during late foetal development, filaggrin accumulates throughout the entire thickness of rat SC and immediately after birth, filaggrin proteolysis occurs in the outer SC.[34]

We found that the cheek skin was a unique site with respect to NMF levels and corneocyte maturity in infancy. NMF levels in cheek SC were much slower to increase compared with other sites, including exposed sites such as the nasal tip (Figure 7). Furthermore, in the first year of life the cheek NMF levels were significantly lower than

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the corresponding levels in the elbow SC. The cheek is frequently the site of initial inflammation in infantile AD. Absolute low levels of NMF and a slow increase in NMF levels may play a role in disease initiation at this site, as may high plasmin activity. The elbow flexure was notable for reaching a steady state of NMF early in infancy. This site is not typically involved in infantile eczema, but is a classic site of childhood AD. The reason why this site is vulnerable in childhood AD is unclear, but may reflect the local microbiome, rather than NMF levels. It has been shown that age strongly affects the microbiome in infants, with bacterial community structure and diversity shifting over time. [35] Furthermore, as compared to exposed skin sites, elbow flexure skin had consistently lower ratio of cis-UCA to total UCA. Cis-UCA has previously been suggested as an immuno-suppressant. [36]

We also report other important insights into post-natal SC biology. In early postnatal life, there is a rapid increase in the ratio between His and its metabolite UCA (Figure 2), which levels off after approximately 3 months. This might be caused by the age- and skin site related differences in the expression and activity of histidase which has the optimum at about pH 8.5 and decreases toward lower pH [37]**.**The pH of the skin surface in the first two weeks after birth is the highest (pH=6.0) and gradually decreases with age.[38]

To further define the generation of NMF in the SC in early post-natal life, as well as SC stress, we measured the activities of SC proteases BH and C-1 and plasmin in a selection of subjects at the cheek and elbow sites. The active forms of BH and C-1 are critical in the final stages of filaggrin degradation.[16-18, 39-41] We demonstrated increased activity of BH in the exposed cheek skin after one month of life. In contrast, levels at the elbow remain unchanged with increasing age. Plasmin activities, which indicate SC

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stress and impaired barrier function, are high in the cheek compared with the elbow at birth. At one month of age plasmin activities increase in the exposed cheek skin, but not at the elbow. Furthermore, plasmin activity correlated with the cis-UCA/tot-UCA ratio in the cheek skin, but not at the elbow. Raj *et al* hypothesised that these atmospheric conditions result in a feedback mechanism to upregulate filaggrin degradation proteases, thereby producing NMF in an effort to improve barrier function and/or epidermal water retention. [31] Our data support this hypothesis, as well as demonstrating that it is relevant in infant skin during early postnatal life.

CE maturity is critical for skin barrier function. We show that the cheek CE is immature from birth and does not improve rapidly with age. Cheek CE immaturity, therefore, is present from birth and persists into adulthood.[5, 31, 42] In adults, facial TEWL is much higher than the forearm and upper arm. [43] It is notable that the facial skin, unlike most other body sites, is continually exposed to environmental stress. The nasal tip is a site of interest in this study; while this site matures rapidly to steady state NMF levels, these levels settle at relatively low levels suggesting that at this site, factors other than NMF or filaggrin expression protect against AD development. At this site sebum production is much higher as is skin hydration, factors that may offset low NMF.

Furthermore, low NMF and low maturity of the CE in the infant cheek SC may be important for allergen sensitization at this site, and subsequent food allergy. There is clear evidence that epicutaneous exposure to peanut through an impaired skin barrier increases the risk of peanut sensitization and confirmed peanut allergy.[44-46] Early life exposure to peanut antigen in household dust is a risk factor for peanut sensitization and allergy in children deficiency [47] and murine models support the concept of an impaired skin barrier as a route for sensitization.[44-46, 48] The

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demonstration of low NMF levels, a more inflammatory plasmin profile and decreased corneocyte maturity in the exposed cheek skin of infants supports the hypothesis of this site being key to food sensitization and allergy in early life, either independently or in the setting of AD. Saliva and food protein exposure causing irritation at this site are likely co-factors in this pathology.

The early postnatal weeks are particularly dynamic for SC maturation. The infant cheek SC has delayed NMF normalisation, higher cohesion and reduced CE maturation, and evidence of increased SC stress from birth. We propose that this combination of factors facilitates the initiation of AD inflammation and allergic sensitisation. Our results support the concept of improving SC moisturisation and skin barrier function in sites of vulnerability in infants in an effort to prevent the onset of AD.[49, 50] Use of moisturisers may compensate for NMF decrease. Even small increases in filaggrin copy number that drive NMF have been shown to be protective against developing AD. [51]

In summary, regional SC biochemical and cellular characteristics may explain the initial clinical patterns of AD in infancy. Cheek SC early in life is likely permissive for development of AD, allergen penetration and food allergy.

Legends to Table & Figures

Table 1. Age groups and sex of all recruited subjects.

Table 2. Sex distribution, means age and age range of all subjects in Phase I and Phase II of study.

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Fig 1. Levels of tot NMF, His, PCA and sum of *trans*- and *cis*-UCA in the SC of children (median with interquartile) in different age groups (< 48 h (n = 18), 48 h – 4 weeks (n = 18), 1 – 11 months (n = 41), 12 – 35 months (n = 25) and 36 – 72 months (n = 30) on three body regions. Differences between age groups were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons test. ***P < 0.001, **P < 0.01 and *P < 0.05.

Fig 2. Ratio of His to UCA (median with interquartile) (panels a, b and c) and linear regression between ratio of His to UCA and age of children (up to 3 months of age; panels d, e and f, from 3 to 72 months of age; panels g, h and i) on three body regions. For regression analysis the values were log-transformed; b=slope of the regression line, CI= 95% confidence interval, r^2 = regression coefficient. ***P < 0.001, **P < 0.01 and *P < 0.05 as determined by Kruskal-Wallis test followed by Dunn's multiple comparisons test. Age groups: < 48 h (n = 18), 48 h – 4 weeks (n = 18), 1 – 3 months (n = 21), 4 – 11 months (n = 20), 12 – 35 months (n = 25), 36 – 72 months (n = 30).

Fig 3. Ratio of *cis*-UCA to *tot*-UCA (*cis+trans*-UCA) (median with interquartile) (panels a, b and c) Differences between age groups were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons test. ***P < 0.001, **P < 0.01 and *P < 0.05. Age groups: < 48 h (n = 18), 48 h – 4 weeks (n = 18), 1 – 3 months (n = 21), 4 – 11 months (n = 20), 12 – 23 months (n = 1), 24 – 35 months (n = 14), 36 – 47 months (n = 12) and 48 – 72 months (n = 18).

Fig 4. Cornified envelope (CE) maturity, SC cohesion, cornecyte surface area and activity of bleomycin hydrolase (BH), calpain-1 (C-1) and plasmin in the SC of children up to 11 months of age (n = 37). Difference between cheek region and elbow flexure region were determined by two-tailed paired t-test (SC cohesion and CE maturity) or by two-tailed

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Wilcoxon matched-pairs signed rank test (corneocyte surface area, BH, C-1 and plasmin). ****$P < 0.0001$, **$P < 0.01$.

Fig 5. Linear regression plasmin activity vs. ratio of *cis*-UCA to *tot*-UCA (*cis+trans*-UCA) on two body sites. The values were log-transformed; b=slope of the regression line, CI= 95% confidence interval, r^2 = regression coefficient.

Fig 6. Cornified envelope (CE) maturity, SC cohesion and cornecyte surface area in the SC of children (median with interquartile) in three age groups, which were; < 48 h (n = 10); 48 h – 4 weeks (n = 10); and 1 – 11 months (n = 16), across two body regions. Differences between age groups were determined by one-way ANOVA followed by Tukey's multiple comparison test (for CE maturity and cornecyte surface area on cheek region Kruskal-Wallis test followed by Dunn's multiple comparisons test was used). *$P < 0.05$.

Fig 7. Cheek skin is slow to mature with respect to NMF levels and corneocyte maturity. While other exposed sites such as the nasal tip rapidly reach steady state maturity in the early months of life the cheek skin starts life with the lowest NMF and only reaches steady state NMF levels at approximately age 7. NMF levels are graphically represented by depth of blue colour.

Legends to supplemental tables and figures

Supplemental Table S1: Demographics of subjects included in SC protease and CE maturity study.

Supplemental Table S2: Filaggrin status of all recruited subjects.

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Supplemental Figure S1. Levels of totNMF in the SC of children (median with interquartile) (panels a, b and c) and regression analysis totNMF vs. age of children (up to 4 weeks of age; panels d, e and f, from 1 to 72 months of age; panels g, h and i) on three body regions. For regression analysis the values were log-transformed; b=slope of the regression line, CI= 95% confidence interval, r^2 = regression coefficient. Differences between age groups were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons test. ***P < 0.001, **P < 0.01 and *P < 0.05. Age groups: < 48 h (*n* = 18), 48 h – 4 weeks (*n* = 18), 1 – 11 months (*n* = 41), 12 – 35 months (*n* = 25) and 36 – 72 months (*n* = 30).

Supplemental Figure S2. Level of totNMF at different SC depth (mean + SEM) in different age groups (< 48 h (*n* = 7) and 48 h – 4 weeks (*n* = 2) and 1 – 11 m (*n* = 4)) on two body regions; cheek and elbow flexure (depth: 2 = 2nd SC tape, 6 = 6th SC tape, 8 = 8th SC tape).

Supplemental Figure S3. Levels of totNMF, His, PCA and sum of *trans*- and *cis*- UCA in the SC of children (median with interquartile) in different age groups, which were: 1 – 11 months (*n* = 25); 12 – 35 months (*n* = 25); and 36 – 72 months (*n* = 27); across four body regions. Differences between age groups were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons test (for His on nape of neck, dorsal upper limb and buttock region One-way ANOVA followed by Tukey's multiple comparison test was used). ***P* < 0.01 and **P* < 0.05.

Supplemental Figure S4

NMF values between C and E during the 1st year of life and for all ages >1 year. T. (Wilcoxon paired 2-sided t-test)

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Supplemental Figure S5. Bleomycin hydrolase (BH), calpain-1 (C-1) and plasmin activities in the SC of children (median with interquartile) in three age groups, which were: < 48 h (n = 10); 48 h – 4 weeks (n = 10); and 1 – 11 m (n = 16); across two body regions. Differences between age groups were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons test (for BH activity on elbow region one-way ANOVA followed by Tukey's multiple comparison test was used). $^{**}P < 0.01$, $^{*}P < 0.05$.

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	Number	Male	Female	Mean Age	Age Range
Phase I	129	78	51	27.9 months	0.25-70 months
Phase II	59	34	25	20.5 days	1 – 84 days
Total	188	112	76	-	-

Age groups		<48 hrs	48hr-4w	1-3m	4-11m	12-36m	36-72m
	All	26	20	26	28	44	43
	Male	13	12	17	14	28	27
	Female	13	8	9	14	16	16

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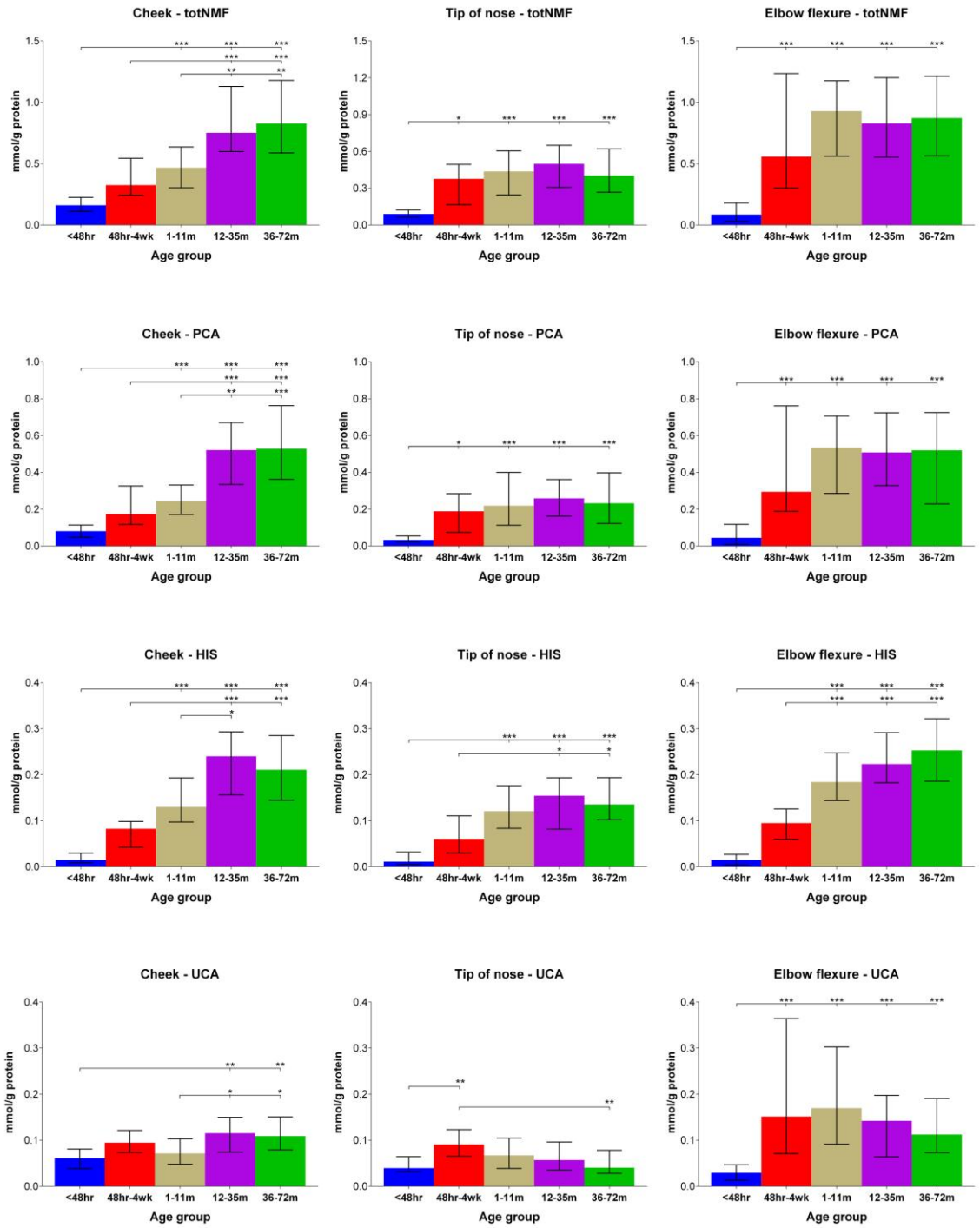

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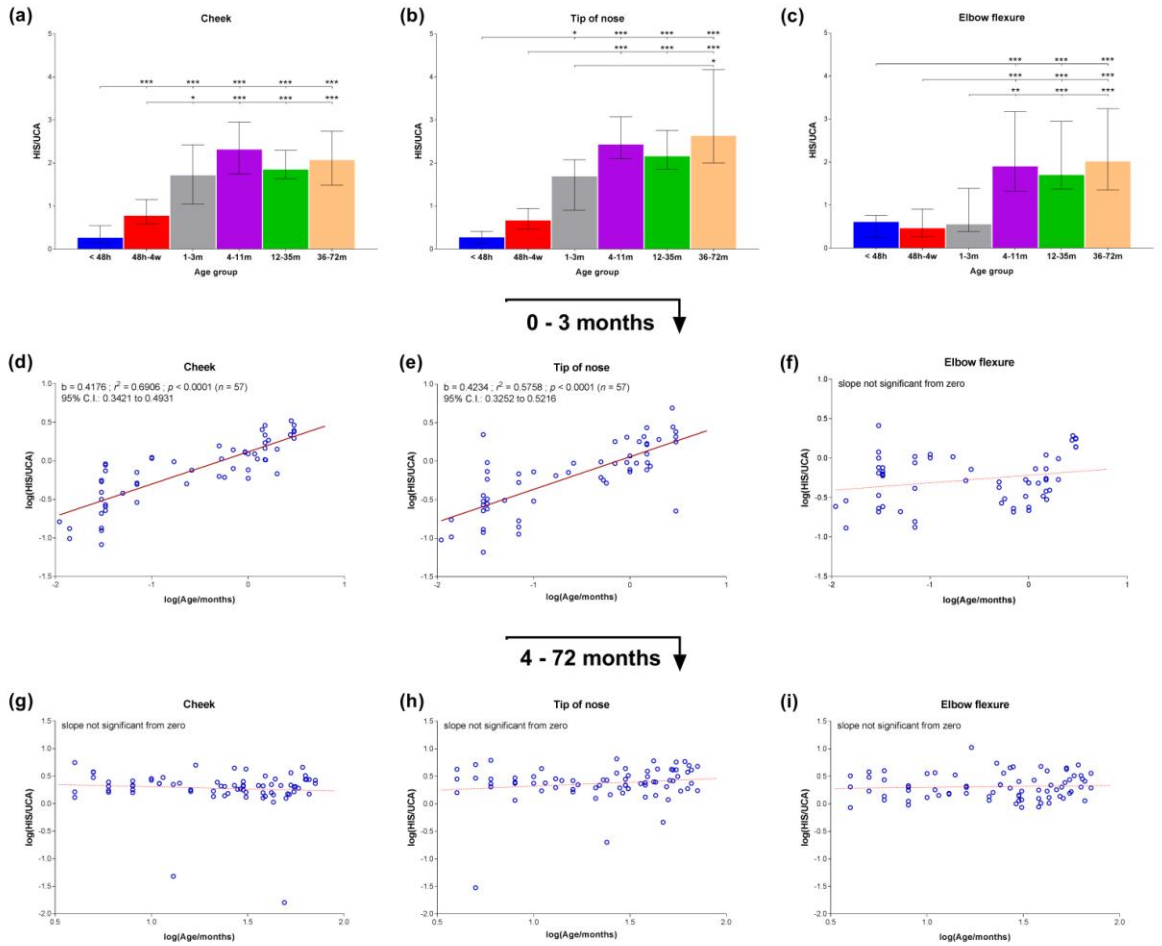

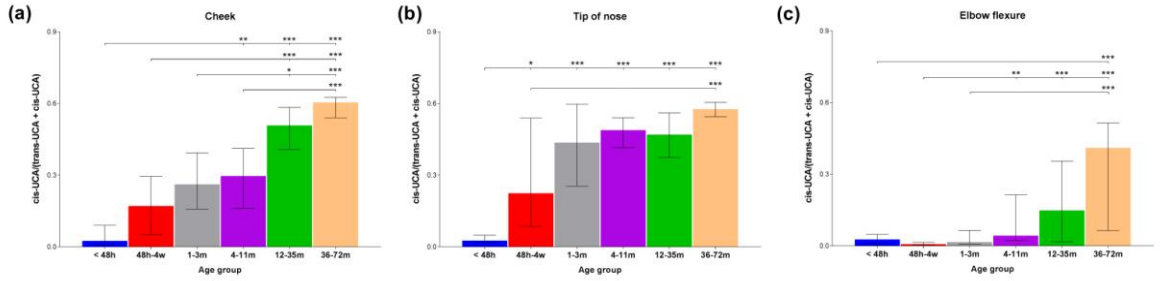

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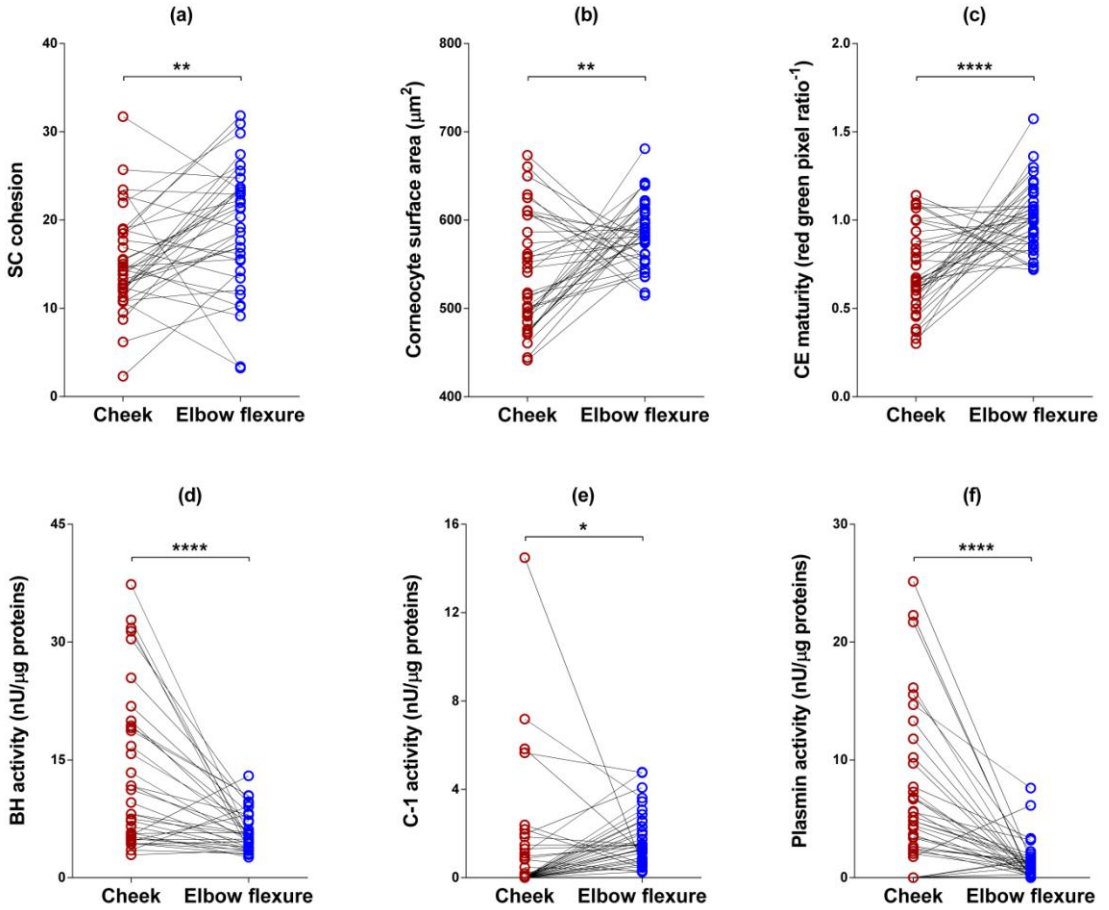

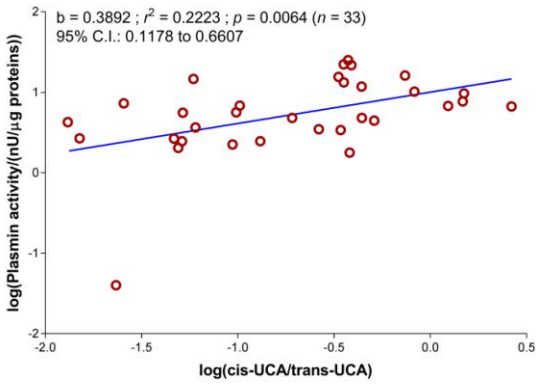

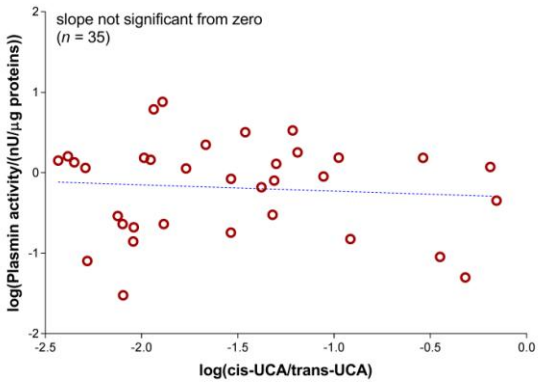

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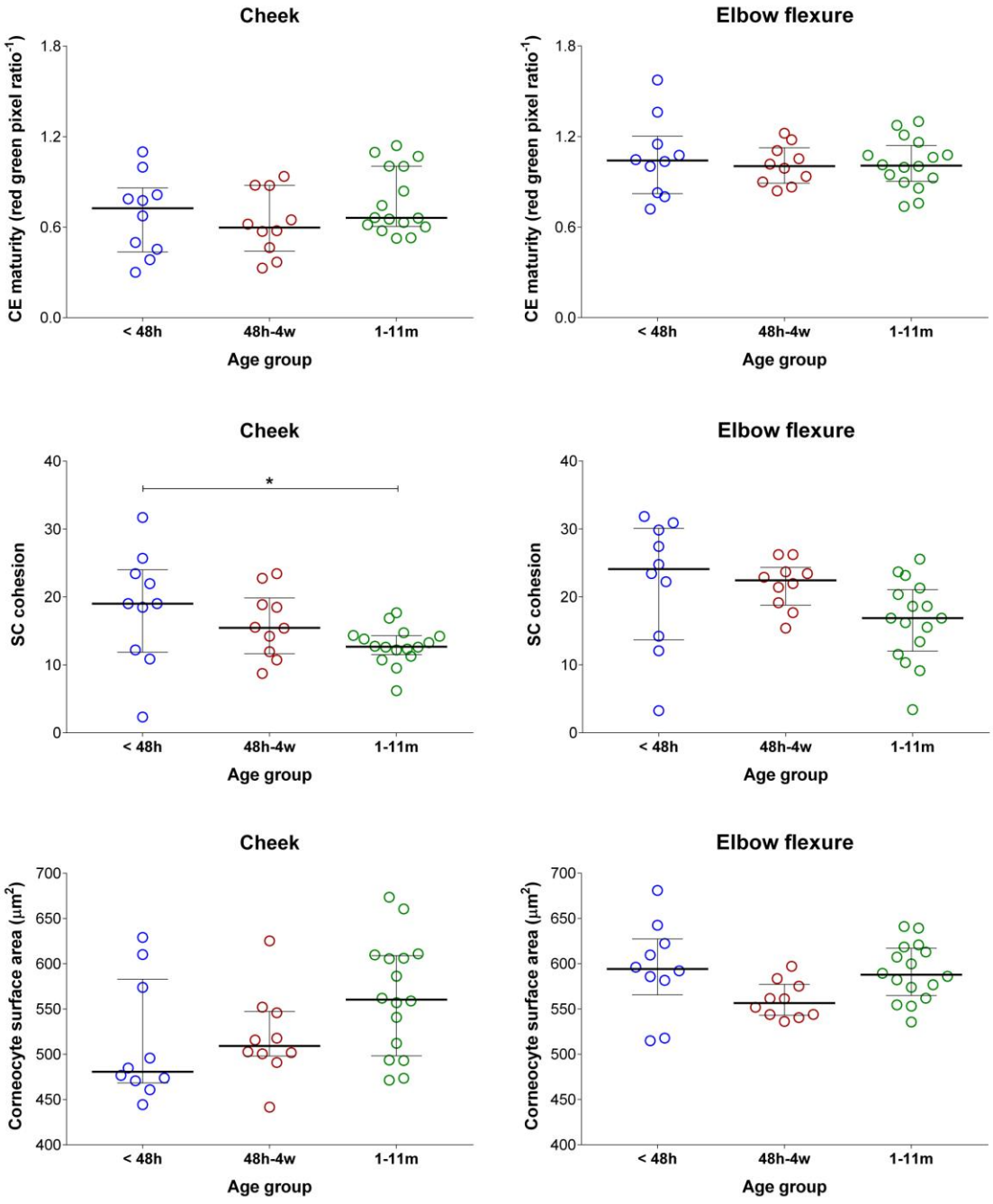

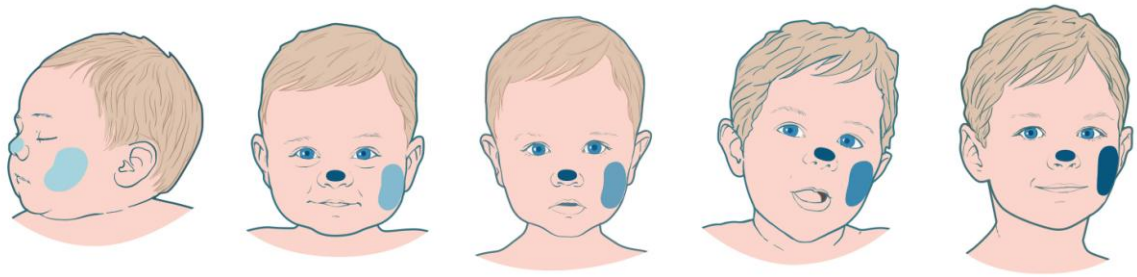

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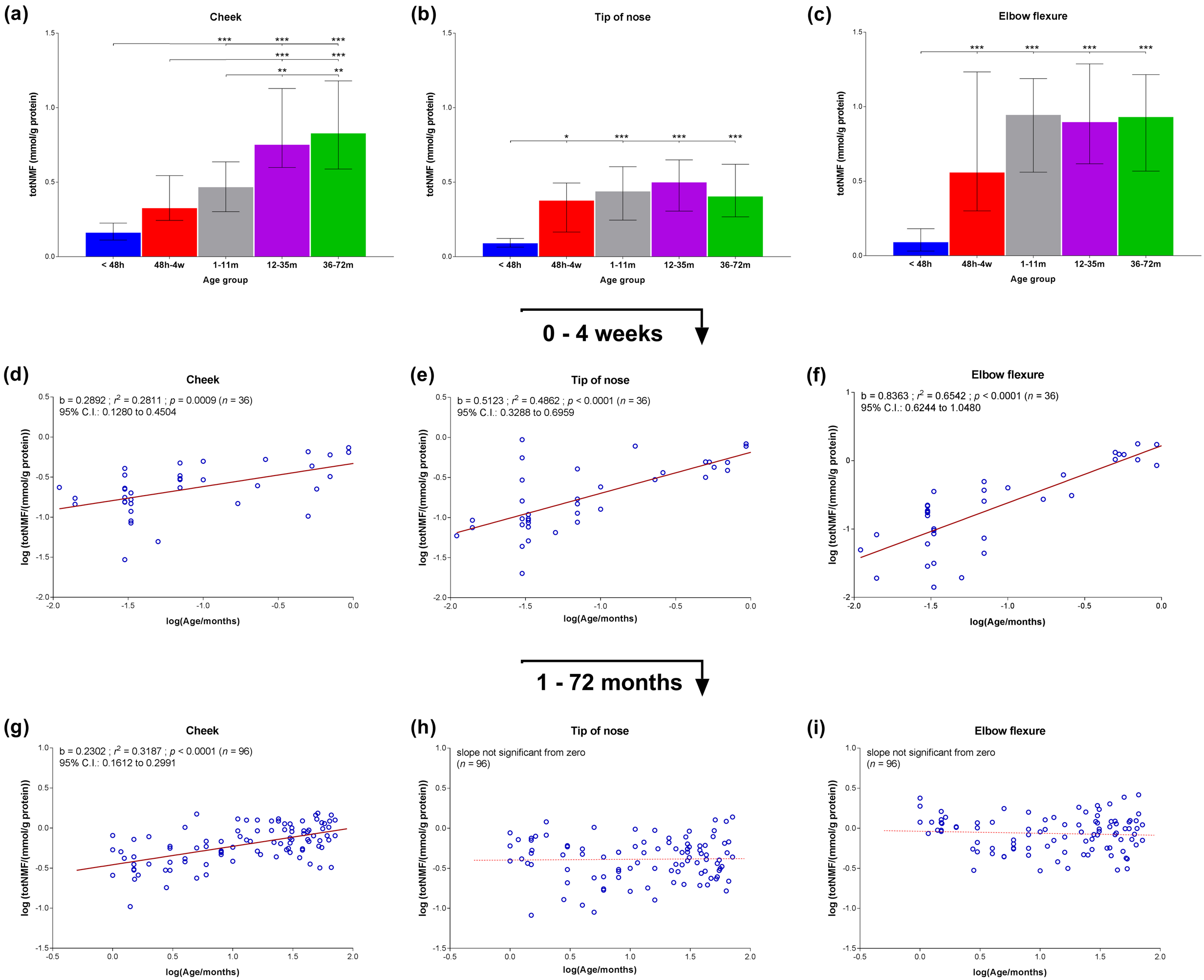
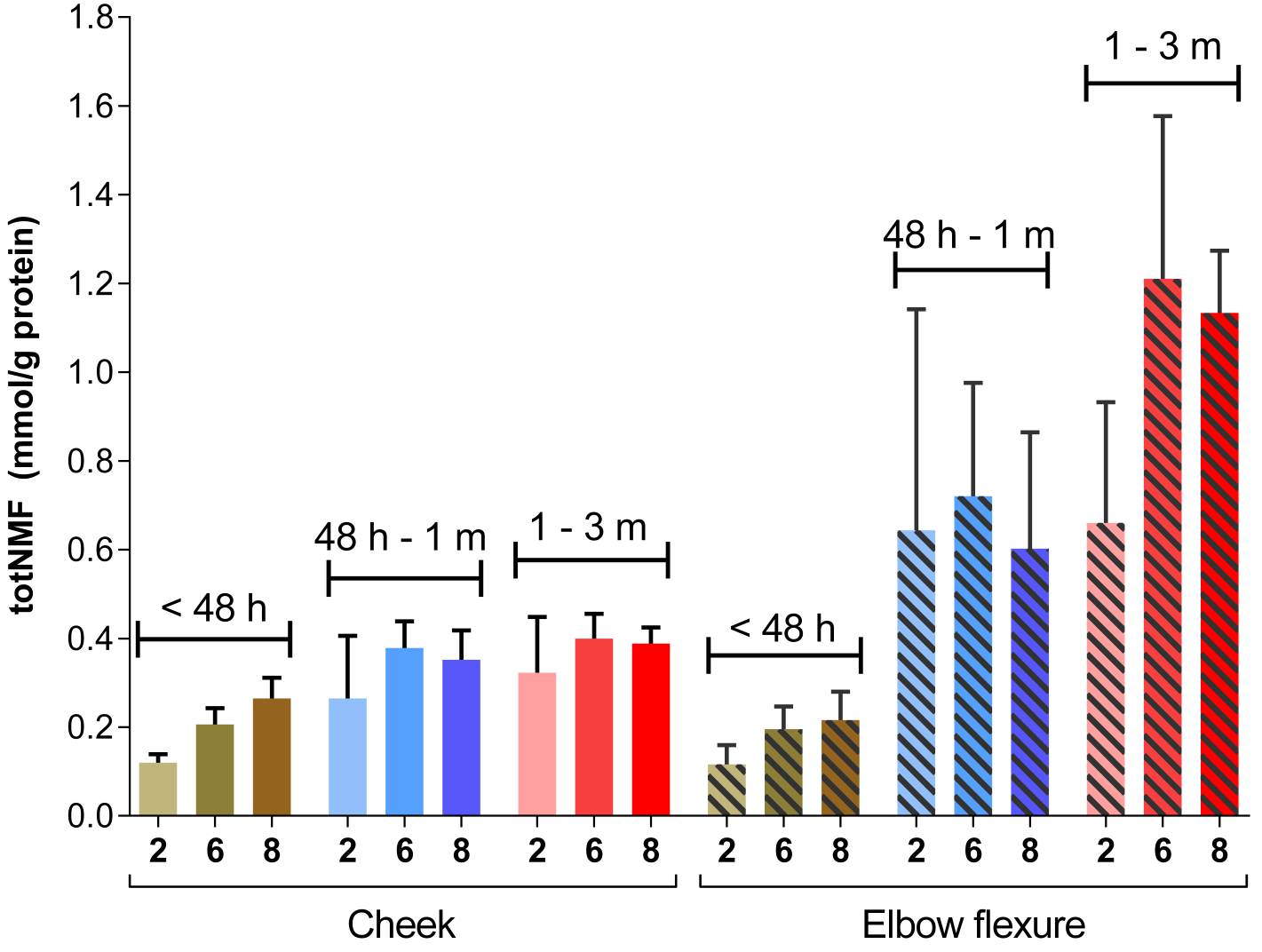
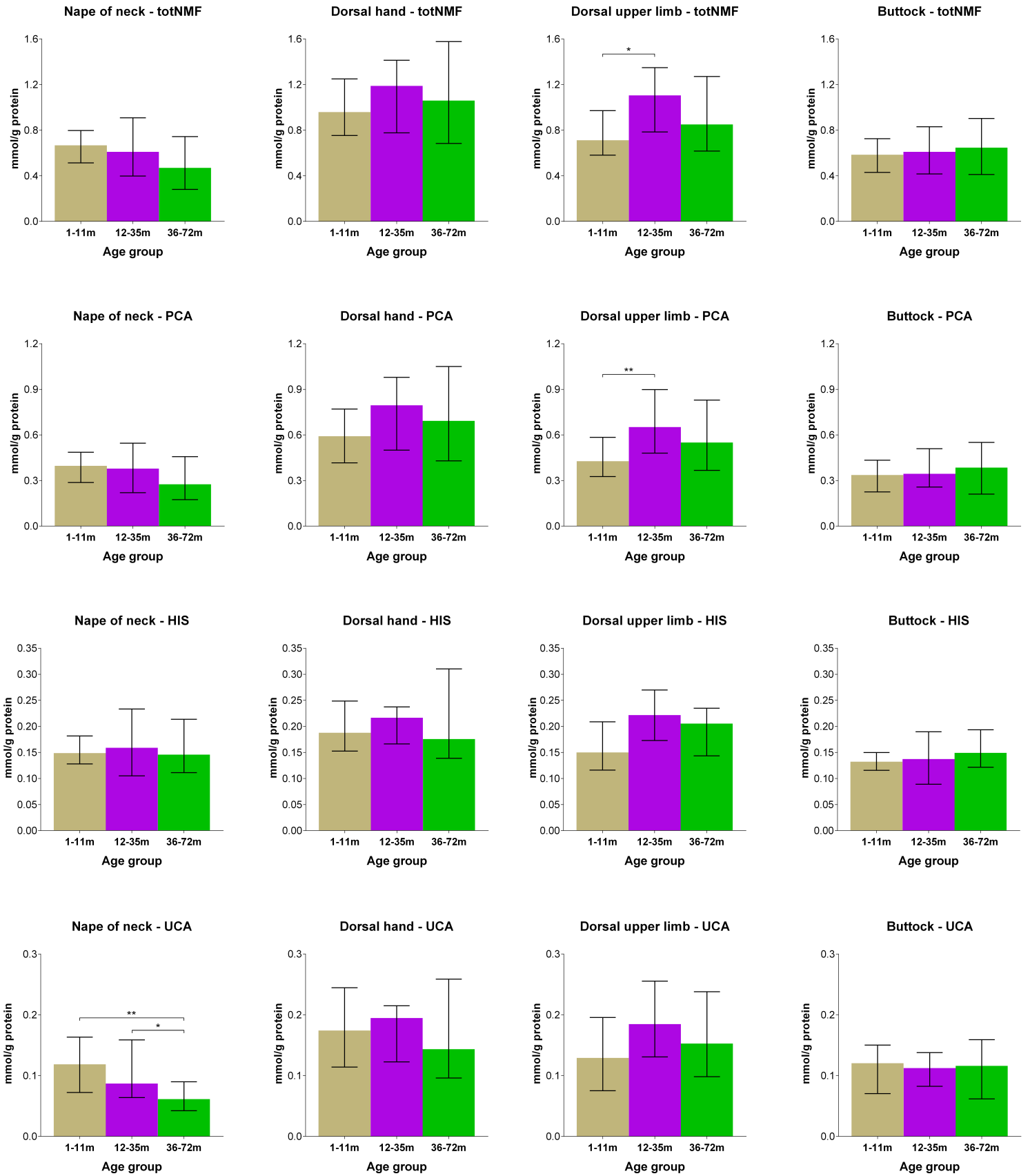
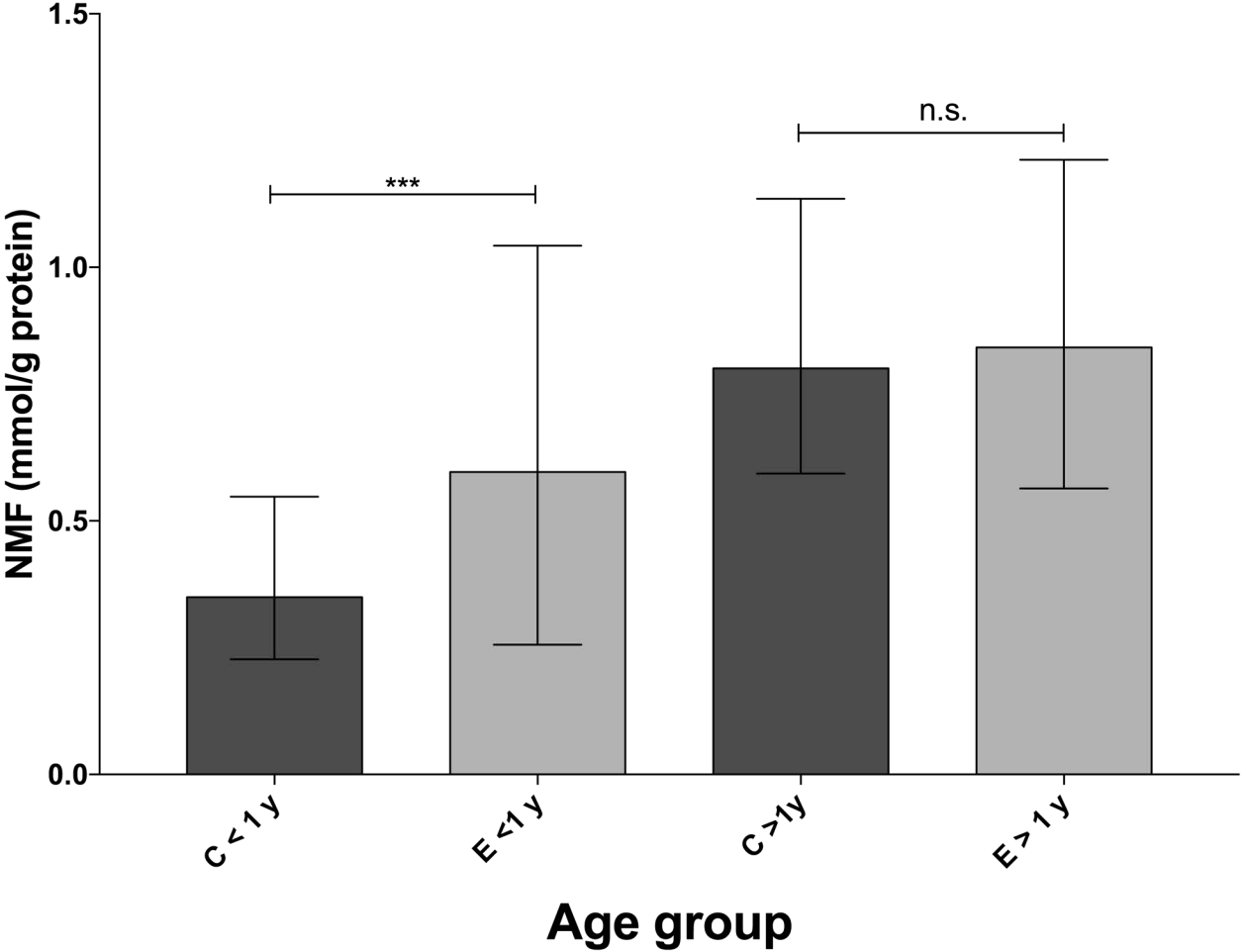
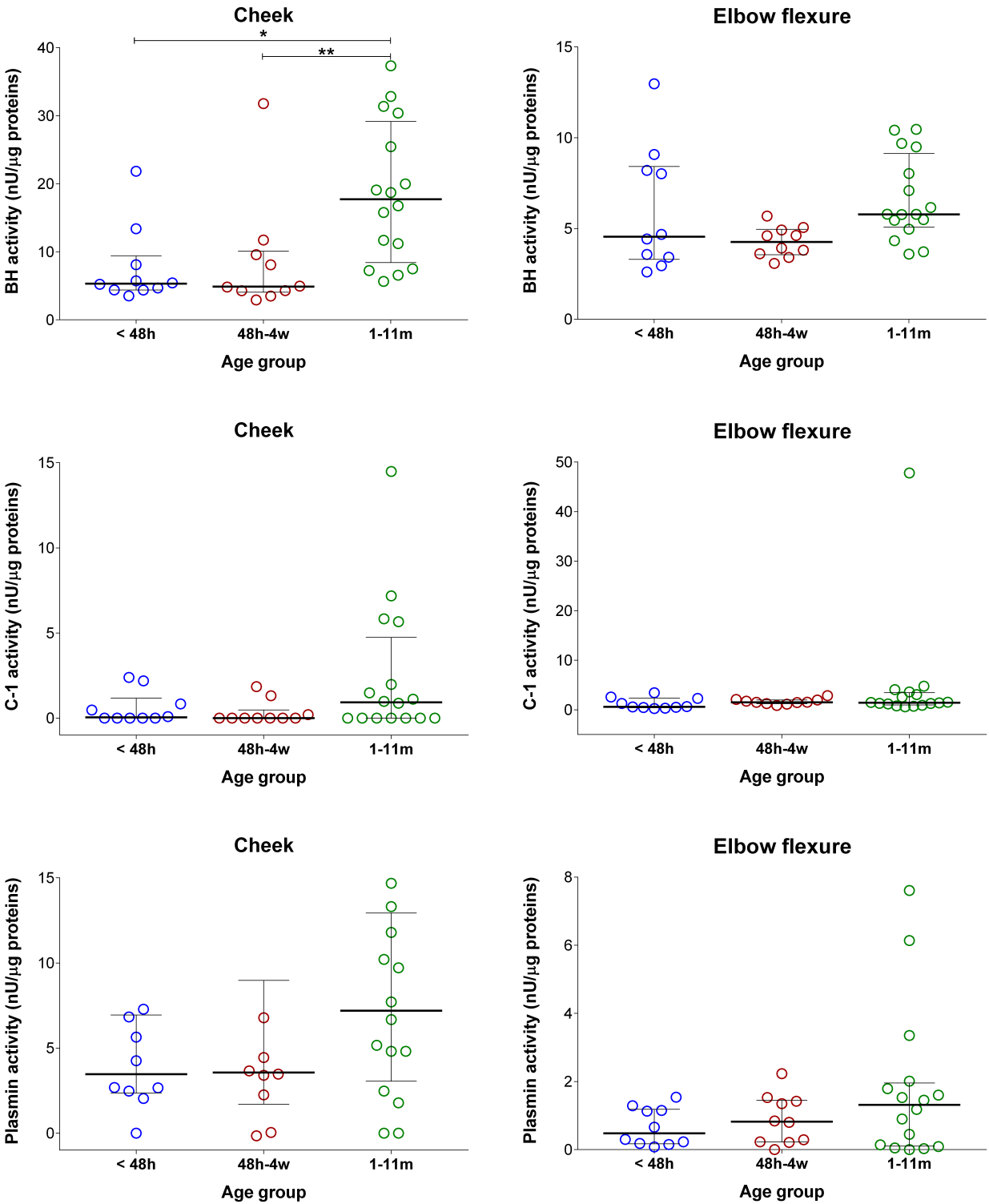

(a)
Cheek
totNMF (mmol/g protein)
Age group
< 48h
48h-4w
1-11m
12-35m
36-72m
(b)
Tip of nose
(c)
Elbow flexure
0 - 4 weeks
(d)
Cheek
b = 0.2892 ; r2 = 0.2811 ; p = 0.0009 (n = 36)
95% C.I.: 0.1280 to 0.4504
log (totNMF/(mmol/g protein))
log(Age/months)
(e)
Tip of nose
b = 0.5123 ; r2 = 0.4862 ; p < 0.0001 (n = 36)
95% C.I.: 0.3288 to 0.6959
(f)
Elbow flexure
b = 0.8363 ; r2 = 0.6542 ; p < 0.0001 (n = 36)
95% C.I.: 0.6244 to 1.0480
1 - 72 months
(g)
Cheek
b = 0.2302 ; r2 = 0.3187 ; p < 0.0001 (n = 96)
95% C.I.: 0.1612 to 0.2991
(h)
Tip of nose
slope not significant from zero
(n = 96)
(i)
Elbow flexure
slope not significant from zero
(n = 96)


totNMF (mmol/g protein)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
< 48 h
48 h - 1 m
1 - 3 m
< 48 h
48 h - 1 m
1 - 3 m
2 6 8 2 6 8 2 6 8 2 6 8 2 6 8 2 6 8
Cheek
Elbow flexure


Nape of neck - totNMF
Dorsal hand - totNMF
Dorsal upper limb - totNMF
Buttock - totNMF
Nape of neck - PCA
Dorsal hand - PCA
Dorsal upper limb - PCA
Buttock - PCA
Nape of neck - HIS
Dorsal hand - HIS
Dorsal upper limb - HIS
Buttock - HIS
Nape of neck - UCA
Dorsal hand - UCA
Dorsal upper limb - UCA
Buttock - UCA
mmol/g protein
1-11m
12-35m
36-72m
Age group
*
**


1.5
1.0
0.5
0.0
NMF (mmol/g protein)
***
n.s.
C < 1 y
E <1 y
C >1y
E > 1 y
Age group


Cheek
*
**
BH activity (nU/µg proteins)
40
30
20
10
0
< 48h
48h-4w
1-11m
Age group
Elbow flexure
BH activity (nU/µg proteins)
15
10
5
0
< 48h
48h-4w
1-11m
Age group
Cheek
C-1 activity (nU/µg proteins)
15
10
5
0
< 48h
48h-4w
1-11m
Age group
Elbow flexure
C-1 activity (nU/µg proteins)
50
40
30
20
10
0
< 48h
48h-4w
1-11m
Age group
Cheek
Plasmin activity (nU/µg proteins)
15
10
5
0
< 48h
48h-4w
1-11m
Age group
Elbow flexure
Plasmin activity (nU/µg proteins)
8
6
4
2
0
< 48h
48h-4w
1-11m
Age group

Age groups	<48 hrs	48hr-4w	1 -3 m	4-11m	All Age Groups
Number	10	10	9	8	37
Mean Age (months)	0.03	0.38	1.78	7.4	2.41
Age range (months)	<0.033-0.03	0.07-0.93	1 - 3	4- 11.5	<0.033-11.5

	FLG WT	FLG HET	Unknown
Phase I	118	11	0
Phase II	46	1	12