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In-vivo Raman spectroscopy discriminates between *FLG* loss-of-function carriers vs. wild-type in day 1-4 neonates

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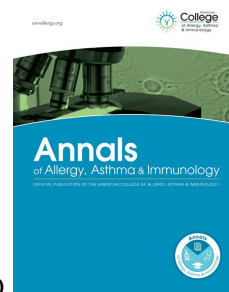
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Title page

Title *In-vivo* Raman spectroscopy discriminates between *FLG* loss-of-function carriers vs. wild-type in day 1-4 neonates

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Conflicts of interest

J O'B Hourihane receives research funding from DBV Technologies.

A Irvine acts as a consultant to Johnson & Johnson

J O'B Hourihane and A Irvine receive research support from Johnson & Johnson

C Nico, G Puppels and P Caspers are employees of RiverD International B.V. which has developed the NMF-scan prototype used in this study and which owns the intellectual property in the field of in-vivo analysis of natural moisturising factor in the skin. J O'B Hourihane is a member of the clinical advisory board for River D International B.V.

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Clinical Trial Registration Not applicable

Keywords

Filaggrin, skin barrier, non-invasive assessments, biomarkers, atopic dermatitis, Raman spectroscopy

Abbreviations

AD – Atopic dermatitis

AUROC – Area under the receiver operating curve

CNV – Copy number variation

FLG – Filaggrin

LoF – Loss-of-function

NMF – Natural moisturising factor

PCR – Polymerase chain reaction

SC – Stratum corneum

SD – Standard deviation

TEWL – Transepidermal water loss

Word count 2052

Figures 5 figures

Figure 1 Study flow-diagram

Figure 2 Raman-derived NMF values across age in days ($n = 139$, 1 day; 25.9%, 2 days; 36.7%, 3 days; 28.1%, 4 days; 9.4%), $^{**}P < 0.05$

Figure 3 Neonatal NMF as a function of age in days and filaggrin (*FLG*) genotype (*FLG* wildtype vs. *FLG* mutation carrier)

Figure 4 Box-plot of Raman-derived NMF content ‘corrected’ to Day of life by filaggrin (*FLG*) genotype ($n = 117$)

Figure 5 ROC curve of neonatal Raman-derived NMF(corrected to Dat 3 of life) sensitivity and specificity in the prediction of *FLG* genotype [AUC (95% CI): 0.83 (0.75, 0.92), $P \leq 0.001$]

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Table 1 Subject characteristics, NMF, TEWL and filaggrin mutation status in those with neonatal NMF measurements ($n = 139$)

Table 2 Filaggrin copy number variants (CNV)

19-12-0602R2 abstract

Background Carriers of loss-of-function mutations in the filaggrin (LoF FLG) gene have less natural moisturising factor (NMF) in their stratum corneum (SC) and an increased risk of atopic dermatitis (AD). NMF can be measured non-invasively by Raman spectroscopy. The use of Raman-derived NMF at birth to screen for FLG genotype could inform targeted AD prevention, but values in neonatal populations are largely unexplored.

Objective To examine the associations between Raman-derived neonatal NMF measurements and FLG genotype.

Methods NMF was measured by Raman Spectroscopy in the SC of the thenar eminence within 4 days of birth in 139 term neonates. FLG genotyping was performed for 117 neonates (84%).

Results The mean (SD) NMF was 0.37 (0.11) g/g protein, with values increasing across the first three days [Day 1 vs. 3: 0.29 (0.09) vs. 0.43 (0.08), $P < 0.001$]. Twelve infants (10.3%) were carriers of LoF FLG, all heterozygous. NMF was lower in LoF FLG carriers compared with wild type [0.27 (0.08) vs. 0.38 (0.11) g/g protein, $P \leq 0.001$]. NMF had good discriminatory power for FLG genotype [area under the receiver operating curve (AUROC): 0.79, 95% CI: 0.66, 0.91, $P \leq 0.001$]. This improved after correcting Day 1 and 2 measurements to Day 3 [AUROC: 0.83, 95% CI: 0.75, 0.92, $P < 0.001$].

Conclusion This study suggests that Raman-derived NMF measured in the early postnatal period may have the potential to classify by FLG genotype. The full translational value of this needs to be determined.

Introduction

Filaggrin deficiency due to loss-of-function mutations in the filaggrin gene (LoF *FLG*), is associated with impaired skin barrier function and plays a central role in the inherited risk of atopic dermatitis (AD) ^{1, 2}. Filaggrin is degraded in the stratum corneum (SC) releasing a mixture of hygroscopic amino acids that collectively make up the majority of natural moisturising factor (NMF), which in turn plays a role in skin barrier function and hydration ³. Carriers of LoF *FLG* mutations have reduced levels of NMF in the SC and thus, NMF could be potentially be used as a surrogate marker for *FLG* genotype ^{4, 5}. Skin NMF can be measured quickly and non-invasively using Raman confocal microspectroscopy ^{5, 6}. In a study of 132 Irish children (aged 4-16 years) with moderate-to-severe AD, Raman-derived NMF concentrations identified subpopulations that corresponded closely with *FLG* genotype, with lower NMF values in those with one or two *FLG* mutations ⁵. In addition to the risk associated with LoF *FLG* mutations, *FLG* intergenic copy number variation (CNV) may also contribute to AD risk ⁷. Allelic variants of the *FLG* gene show between 10-12 almost identical tandem repeats of the filaggrin protein encoded in exon 3 ⁸. Thus, *FLG* CNV varies within a population, which may influence filaggrin expression. In an Irish paediatric population, CNV within the *FLG* gene was compared between 696 cases with AD and 629 controls (no AD), where *FLG* mutation carriers were excluded ⁷. The control group had a significantly higher number of repeats than the AD cases, with a reduced odds ratio of AD for each additional unit of copy number. The same study reported a small positive correlation between urocanic acid, a filaggrin breakdown product and CNV in 31 AD cases who provided tape-stripped samples of the SC. These findings indicate that *FLG* CNV may determine the quantity of filaggrin expressed in the skin and in turn influence AD risk, independent of *FLG* genotype. No study has examined the correlation between Raman-derived NMF and *FLG* CNV. As AD typically begins early in life, the use of Raman-derived

NMF at, or shortly after, birth as a screening tool for *FLG* genotype and potentially CNV could inform early, targeted prevention of AD in a high-risk subgroup. However, studies that have examined neonatal NMF values have only measured small groups and none have reported associations with *FLG* genotype⁹⁻¹¹. Here, we report the distribution of NMF concentrations in the first 4 days of life and their association with *FLG* genotype and *FLG* CNV.

Methods

Participants were recruited from the postnatal wards of Cork University Maternity Hospital in Cork, Ireland. NMF (g/g of protein) was measured at a depth of 20 $\pm$ 5 μ m in the SC of the thenar eminence within 4 days of birth in full-term neonates ($\geq$ 37 weeks' gestation) using Raman spectroscopy. The instrument used was a prototype Raman spectroscopy system designed specifically for the non-invasive *in-vivo* determination of NMF concentrations of the thenar eminence (NMF-scan, RiverD International B.V., Rotterdam, The Netherlands). The instrument determines the NMF value following the method described by Caspers et al. (2001), using the following conversion: 1 arb unit = 0.407 g/g protein¹². Raman spectra were recorded with a 785-nm infrared laser, with a maximum laser power of 30 mW. The instrument is a class 1M laser device and is both eye- and skin-safe, that does not cause any local effects such as erythema or swelling. An average of 22 Raman spectra at different locations of the thenar eminence were measured per infant. NMF concentrations from the individual Raman spectra were averaged to calculate the mean NMF concentration for each participant. Of the 150 participants recruited to the study, 139 (93%) had successful NMF measurements (**Figure 1**). Neonatal transepidermal water loss (TEWL) was measured on the volar forearm using a closed chamber system vapometer (Delfin Technologies, UK). Infants were acclimatized to the room conditions [mean (SD) temperature and humidity: 24 (1) °C and 49 (7) %, respectively] for at least ten minutes before TEWL measurements were taken.

Buccal swabs were collected from 118 neonates using Oragene DNA OG 250 saliva kits (DNA Genotek, Ontario, Canada) for the analysis of *FLG* genotype. *FLG* genotyping was performed using Microfluidics PCR for full coverage of *FLG* repeat alleles using a method previously described by Wong et al. (2018)¹³. Of the 118 DNA samples taken, one sample failed to amplify on PCR. One-hundred and seventeen samples were successfully genotyped. *FLG* CNV of repeats 8 and 10 was determined using relative coverage-based metrics ($n = 116$). All identified mutations were validated with Sanger Sequencing.

Results

Neonatal NMF and FLG genotype

Subject characteristics are presented in **Table 1**. Neonatal NMF measurements in the first 4 days of life showed a normal distribution. The mean (SD) NMF value was 0.37 (0.11) g/g protein and values ranged from 0.11 to 0.59 g/g protein. NMF concentrations increased with age across the first three days of life [Day 1 vs. Day 3: 0.29 (0.09) vs. 0.43 (0.08) g/g protein, $P < 0.001$] (**Figure 2**). The mean neonatal TEWL (SD) value was 8.10 (5.31) g_{water}/m²/h (range: 2.53 to 32.43 g_{water}/m²/h) with no significant correlation between NMF and TEWL values [Spearman's Rho (r): 0.023, $P > 0.05$].

Twelve infants (10.3%) were carriers of LoF *FLG* mutations, all of whom were heterozygous for the mutation. There was no significant difference in TEWL between LoF *FLG* carriers and wildtype [median (IQR): 7.41 (1.87, 12.31) vs. 6.93 (4.53, 9.73) g_{water}/m²/h, $P = 0.590$]. NMF concentrations were significantly lower in LoF *FLG* carriers compared with wild type [0.27 (0.08) vs. 0.38 (0.11) g/g protein, $P \leq 0.001$] (**Figures 3 and 4**). A receiver operating characteristic (ROC) curve was constructed to examine the predictive ability of Raman spectroscopy to stratify neonates by *FLG* genotype (**Figure 5**). Raman-derived NMF had good discriminatory power for *FLG* genotype in neonates [area under the ROC curve

(AUROC): 0.79, 95% CI: 0.66, 0.91, $P \leq 0.001$]. Based on ROC curve analysis, the optimal cut-off value for mean NMF in the first 4 days of post-natal life to distinguish by *FLG* genotype was 0.32 g/g protein, with a sensitivity of 83% and specificity of 74%.

Levels of NMF were shown to increase over the first three days of life which reflects rapid post-natal processing of filaggrin monomers following transition from the aqueous intrauterine environment to the dry post-natal environment. This represents a potential confounding factor in the association between neonatal NMF and *FLG* genotype so a linear fit of the average NMF in the first three days was constructed to account for this potential confounding. This resulted in a slope of 0.0723 g/g protein per day. Taking this to be the “build-up rate” of NMF over this period, NMF measurements taken at Day 1 and Day 2 were “corrected” to Day 3 [measured NMF + 0.0723*(3-Age in days)] and associations with *FLG* genotype were re-examined. After this correction, there was no significant difference in NMF across age in days. NMF was still significantly lower in LoF *FLG* carriers compared with wild type [Mean (SD): 0.34 (0.07) vs. 0.44 (0.09) g/g protein, $P \leq 0.001$]. The AUROC improved after correction to 0.83 [95% CI: 0.75, 0.92, $P < 0.001$]. The optimal Day 3 NMF cut-off from the ROC analysis was 0.40 g/g protein with a sensitivity of 92% and specificity of 70%.

Neonatal NMF and FLG copy number variants

The frequency of the 10, 11 and 12 repeat alleles in this population was 54.4, 32.8 and 12.7%, respectively. The frequencies of total CNV are presented in **Table 2**. Associations between CNV and NMF were examined excluding *FLG* mutation carriers. There was no significant correlation between Raman-derived NMF and total *FLG* CNV [Spearman’s rho (r): 0.110, $P = 0.272$]. There was still no significant correlation with total CNV when NMF values were corrected to Day 3 [$r = 0.173$, $P = 0.083$].

99 Discussion

100 This is the largest study to describe Raman-derived NMF concentrations in a neonatal
101 population. Neonatal NMF values were lower than values reported in older children aged 4-
102 16 years with moderate-to-severe AD⁵. Skin physiology is dynamic in the early postnatal
103 period, as demonstrated by the increasing NMF concentrations across age in days in this
104 study. In fact, NMF concentrations have been shown to be stabilizing and to demonstrate
105 regional differences over the first year of life^{10, 14}. NMF levels during the newborn period are
106 additionally influenced by the major environmental change of skin adaption from the aqueous
107 intrauterine environment to the dry extrauterine environment. In rats, filaggrin proteolysis
108 was found to only begin after birth, with filaggrin accumulating throughout the entire
109 thickness of the SC during late fetal development³. Despite this, we have demonstrated that
110 Raman-derived NMF concentrations measured in the first 4 days of life can differentiate
111 between LoF *FLG* mutation carriers and wildtype with significantly reduced levels of NMF
112 in carriers of LoF *FLG* mutations. This predictive ability increased after the observed
113 increase in NMF across the first three days of life was accounted for. LoF *FLG* gene
114 mutations are not only associated with the incidence of AD, but also with the severity and
115 persistence of the disease¹⁵. Early screening for *FLG* gene status may allow for targeted
116 prevention of the specific subtype of AD associated with LoF *FLG* mutations.

117 We did not find a significant correlation between Raman-derived NMF and *FLG* CNV. In
118 their study of 31 tape-stripped children with AD, Brown and colleagues reported a small
119 positive association between *FLG* CNV and urocanic acid, with CNV accounting for ~18%
120 of the variation in this NMF component. Positive correlations with two other filaggrin
121 breakdown products, histidine and pyrrolidone-5-carboxylic acid were not significant⁷. The
122 NMF-scan instrument used in our study was not designed to measure individual constituents

of NMF. Additionally, the early postnatal period may be too early to detect any associations between NMF and *FLG* CNV, given that NMF concentrations are still developing in the SC.

Despite LoF *FLG* corresponding to a low NMF, there was wide inter-individual variation within the two genetic groups, with low NMF values also observed in the wild-type group, even after correcting NMF values to Day 3. This variation within the wild type and mutation groups may reflect other genetic and environmental factors affecting filaggrin expression. Small gene deletions and LoF mutations in other epidermal differentiation complex genes would not have been detected by our genetic analysis approach. Raman spectroscopy is therefore potentially more useful than genetic analysis, as it measures the functional consequences of all potential mutations and mechanisms affecting NMF production.

This study has shown that Raman-derived NMF measurements may allow for classification by *FLG* genotype in the early postnatal period, potentially representing a much faster bedside option for screening than genotyping. This was a small methodological study that we believe shows the promise of this technique. We acknowledge that the small sample size and therefore the size of the LoF *FLG* group in this study are limitations. There was also no follow-up of infants to determine a diagnosis of AD. Thus, the clinical utility of identifying those with a low NMF still needs to be determined. We know that LoF *FLG* confers a strong genetic susceptibility to AD, with an approximately 3.12 times increased risk of development of any AD and an increased risk of a more severe and persistent AD phenotype¹⁶. In comparison, the strongest other known risk factor, parental history of atopy, confers a 3.3 times increased risk of AD¹⁷. While *FLG* mutations do not predict AD development with 100% accuracy, non-invasive and quick screening, using Raman spectroscopy, may identify an at-risk population who may benefit from early intervention. Future larger studies with serial measurements during infancy and long-term follow up should examine whether the predictive accuracy of neonatal NMF for *FLG* status increases over the longer neonatal

148 period so that a threshold value can be established. Standardisation of the best day of life to
149 measure NMF, taking into account both the predictive accuracy and the opportunistic
150 window of time that an infant is in hospital after birth could optimise the utility of this
151 technique were it to be widely rolled out. We are currently undertaking one of the further
152 longer-term studies that will be necessary to establish the full translational value of neonatal
153 Raman measurements by determination of whether these measurements predict development
154 of AD, asthma and associated atopic comorbidities.

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Figure legend

Figure1 Study flow-diagram

Figure 2 Raman-derived NMF values across age in days ($n = 139$, 1 day; 25.9%, 2 days; 36.7%, 3 days; 28.1%, 4 days; 9.4%), $**P < 0.05$

Figure 3 Neonatal NMF as a function of age in days and filaggrin (*FLG*) genotype (*FLG* wildtype vs. *FLG* mutation carrier)

Figure 4 Box-plot of Raman-derived NMF content 'corrected' to Day of life by filaggrin (*FLG*) genotype ($n = 117$)

Figure 5 ROC curve of neonatal Raman-derived NMF(corrected to Dat 3 of life) sensitivity and specificity in the prediction of *FLG* genotype [AUC (95% CI): 0.83 (0.75, 0.92), $P \leq 0.001$]

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Table 1 Subject characteristics, NMF, TEWL and filaggrin mutation status in those with neonatal NMF measurements (*n* = 139)

	NMF measured (<i>n</i> = 139)
Sex [% (n) female]	47.5 (66)
Gestational age (weeks) [Mean (SD)]	39.7 (1.0)
Age in days [mean (SD)]	2.2 (0.9)
Age [% (n)]	
1 day	25.9 (36)
2 days	36.7 (51)
3 days	28.1 (39)
4 days	9.4 (13)
Mean (SD) NMF	0.37 (0.11)
Mean (SD) TEWL	8.10 (5.31)
<i>FLG</i> mutation carrier, 117 measured [%, (n)]	10.3 (12)

SD; standard deviation, NMF; Natural moisturising factor, TEWL; transepidermal water loss, *FLG*; Filaggrin

Table 2 Filaggrin copy number variants (CNV)

<i>FLG</i> Total copy number	Total group n (%), <i>n</i> = 116	Excluding <i>FLG</i> mutation carriers (n,%), <i>n</i> = 104
20	42 (36.2)	37 (35.6)
21	42 (36.2)	37 (35.6)
22	8 (6.9)	7 (6.7)
23	21 (18.1)	20 (19.2)
24	3 (2.6)	3 (2.9)

CNV; copy number variants, *FLG*; Filaggrin

Figure 1

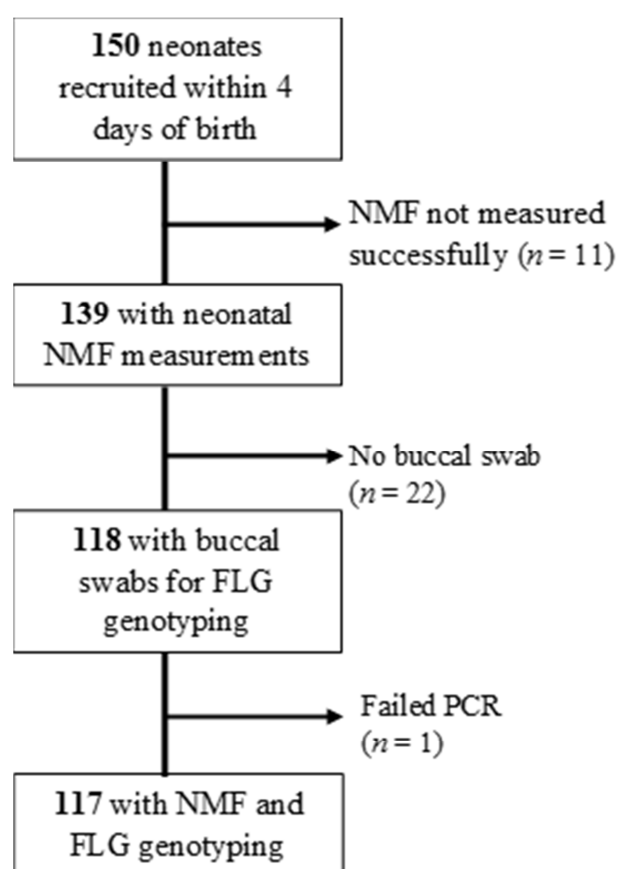


Figure 2

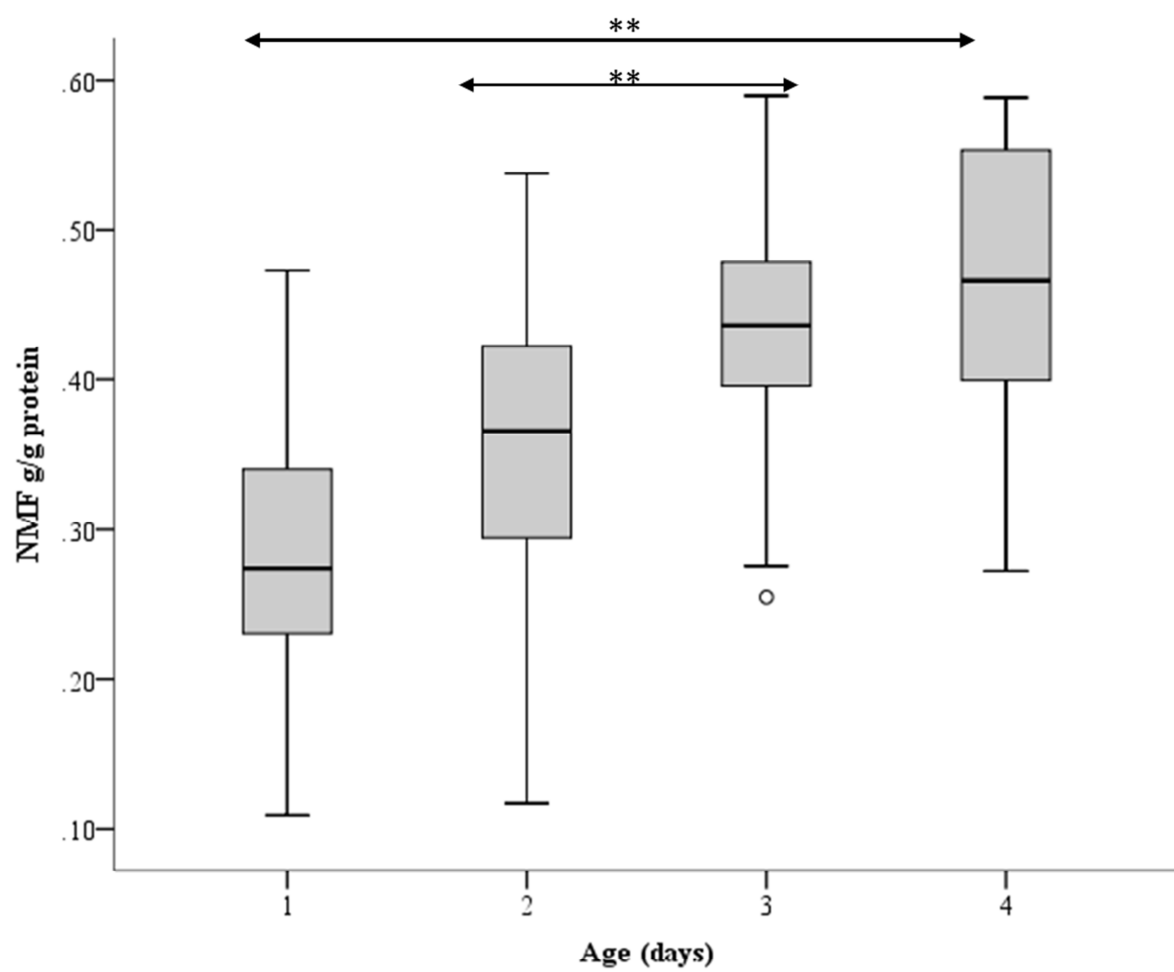


Figure 3

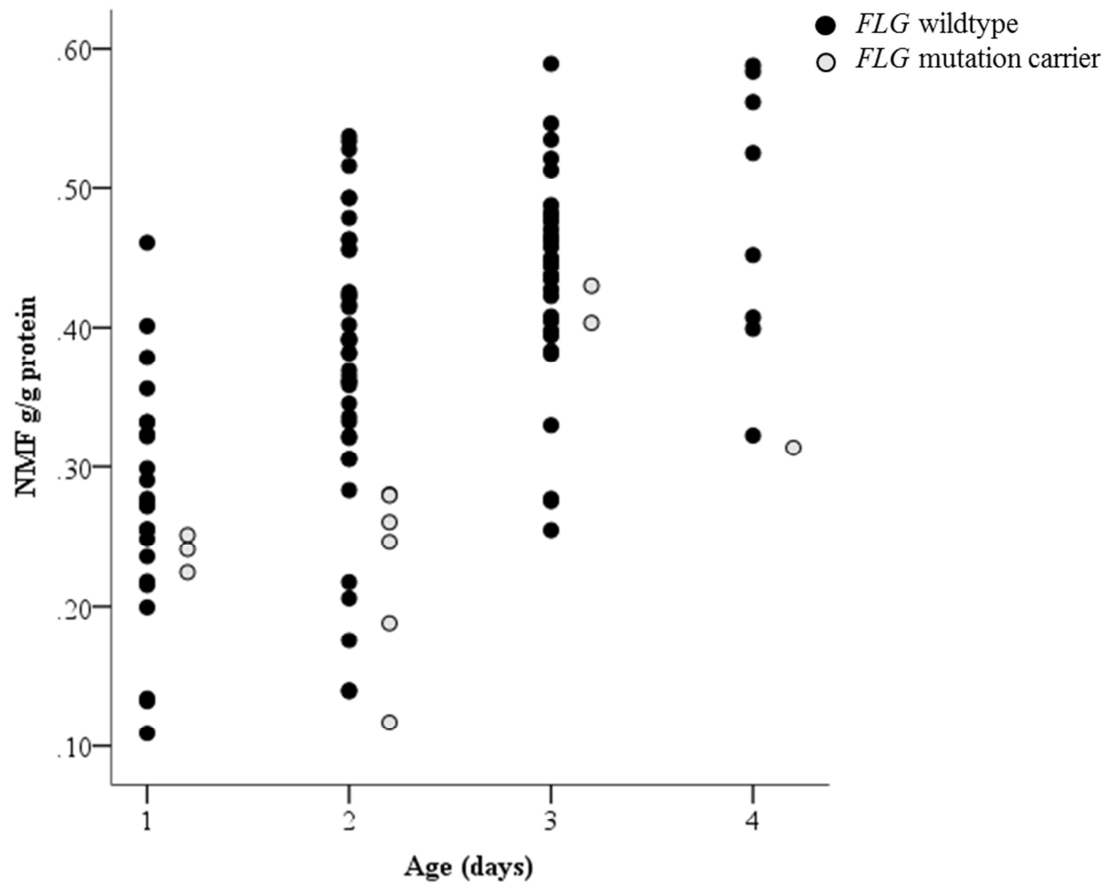


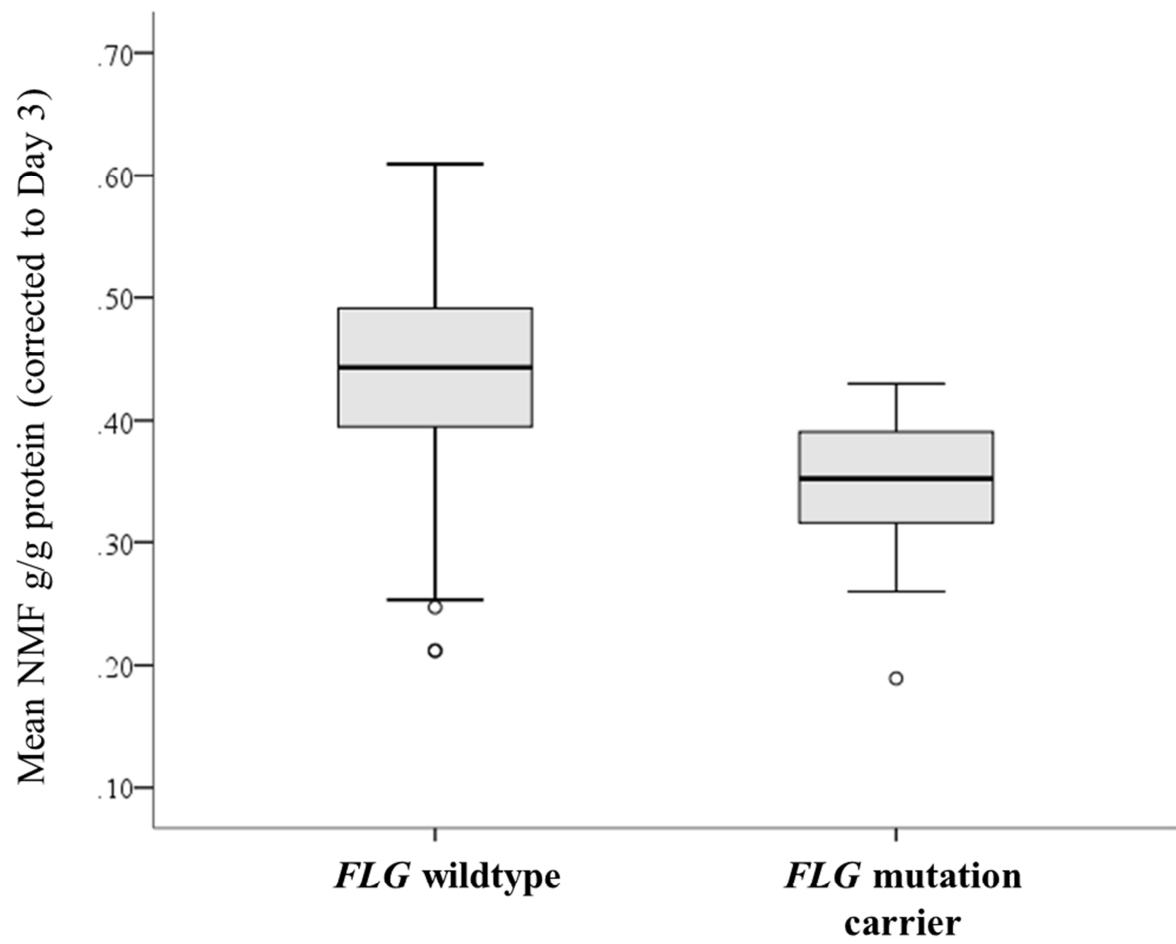
Figure 4

Figure 5