

# Stability of faecal hepatocyte growth factor determination

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In order to evaluate the accuracy and reproducibility of determination of hepatocyte growth factor (HGF) levels in faeces, the stability of HGF in samples processed in different ways was investigated. An ELISA method was used for determination of HGF concentrations. Faeces samples from healthy controls and patients with infectious diarrhoea were studied. It was found that faeces HGF concentration remained stable irrespective of whether samples were freeze-thawed several times, kept for 6, 12 or 24 h at room temperature or refrigerated for 6, 12, 24 or 36 h; the levels of HGF did not change significantly when samples were freeze-dried. Adding protease inhibitor to the faeces samples did not affect the HGF levels. There were no significant differences between HGF levels using phosphate buffered saline (PBS) (pH 7.4) or NaCl as buffer, but it was observed that levels of HGF were significantly lower in the samples that were diluted in distilled water. Although both HGF and albumin through various mechanisms may increase in faeces during infectious diarrhoea, there was no significant correlation between faeces HGF levels and albumin levels, which might indicate local production of HGF in the bowel in response to infection. It is concluded that determination of faeces HGF levels is feasible with a high degree of stability. Increased HGF levels in faeces might represent a local production of HGF during bowel injury and might be of use as a diagnostic and monitoring assay.

*Key words:* faeces; hepatocyte growth factor; protease inhibitor; stability

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## INTRODUCTION

The multifunctional cytokine hepatocyte growth factor (HGF), has been studied in various diseases causing organ injury [1–3]. Produced in high amounts both systemically

and locally, HGF fulfils essential functions in order to repair the damaged organ [4–6]. High systemic concentrations of HGF during diseases such as acute infectious diseases [7, 8], pancreatitis [9], hepatitis [10] and acute renal failure [11] might depend on the induced

expression of HGF mRNA in the intact organ in response to injuries in the damaged tissue [12]. HGF is produced locally at the site of injury [13] and might interact with epithelial cells in a paracrine fashion [5]. We previously studied the levels of HGF during different infectious diseases [7]. Recently, we found high levels of HGF in faeces during transmittable diarrhoea [14]. Investigation of both the method of handling and the stability of substances in various biological samples is important for the planning of comparable and reliable studies. The stability of HGF in blood samples [15] and in exhaled breath condensate (unpublished data) has been investigated by our group. The aim of the present study was to investigate faeces samples in order to establish a standard method of sample handling.

## PATIENTS AND METHODS

### *Patients*

Faeces samples from 8 patients with acute infectious gastroenteritis and without severe renal or liver disease (3 F, 5 M, aged 18–49 years) were used. The faeces cultures yielded growth of *Salmonella* group D in 3 patients and *Campylobacter pylori* in 4 patients. The faeces culture was negative in one patient who had contact with other cases of diarrhoea in his family during the same time period. For comparison, faeces samples from 8 healthy volunteers (5 F, 3 M, aged 22–44 years) were used.

Stool samples from a second patient group were reassessed. Stool from 23 patients (13 F, 10 M, aged 16–83 years) had been collected 2 years earlier. This group consisted of 17 patients with acute gastroenteritis, 2 patients with active Crohn's disease and 4 patients with Crohn's disease in remission. Nine of the 17 patients with acute gastroenteritis came to a follow-up visit 4–6 weeks later. Thus, in this second group, 32 faeces samples from 23 patients were reconstituted (see below). These samples had been analysed 2 years earlier and then stored at  $-20^{\circ}\text{C}$  (the same reconstituted sample which was thawed once and kept frozen), and were reassessed in the present study (substudy 10). The study was approved by the local committee of research ethics in Linköping.

### *Determination of HGF in faeces*

*Standardizing faeces volume and reconstitution procedure.* A simple method was used to standardize the process of obtaining the exact volume of faecal samples. Faeces samples were collected from patients and stored within one hour at  $-20^{\circ}\text{C}$ . Prior to handling, the samples were thawed at room temperature and mixed using a Vortex (Vortex-Genie, Scientific Industries Inc., Bohemia, NY, USA). The narrow heads of plastic syringes (Omnifix 2 mL, latex free, B. Braun Melsungen AG, DE-34209 Melsungen, Germany) were cut off. The plungers of the syringes were pulled out to create small cylinders with an exact volume. The cylinders were filled using a wooden stick and object glasses were placed on top of the cylinders. The samples were then placed at  $-70^{\circ}\text{C}$  for 15 min followed by room temperature for 1 min to facilitate moving of faeces in the syringe. For the substudies employing buffer solutions, the plunger of the syringe was then pushed to empty the cylinder into a flask (scintillation vial 20 mL, Sarstedt AB, Landskrona, Sweden) containing the respective buffer at a dilution of 1:6. The flask was then mixed again using a Vortex. The suspension was centrifuged at  $1000\text{--}3000\times g$  in 15 min and the supernatant was transferred to tubes (Nunc Cryo Tube, Nunc Brand Products, Denmark) and stored at  $-70^{\circ}\text{C}$  pending analysis. This procedure was employed for the substudies 3–9.

*Determination of HGF.* After storage all samples were thawed and centrifuged at  $1000\times g$  for 15 min prior to analysis. Immunoreactive HGF was determined by ELISA using a commercially available kit (Quantikine HGF Immunoassay, R&D Systems Inc., Minneapolis, USA). The minimum detectable limit was determined by adding two standard deviations to the mean optical density value of 70 extracts from the eight faecal samples obtained from the healthy controls and calculating the corresponding concentration, giving  $0.035\ \mu\text{g/L}$ . As positive control, recombinant human HGF (a gift from Professor Toshikazu Nakamura, Osaka University Medical School, Japan) in different concentrations was used.

*Determination of albumin in faeces.* An immunoturbidimetric method (IMMAGE<sup>®</sup> Immunochemistry Systems, Uppsala, Sweden) was used for determination of albumin in faeces. The lowest detectable amount was 6 mg/L faeces.

*Determination of faeces haemoglobin.* The HemoCue system (HemoCue Ltd., Derbyshire, England) is a method that measures haemoglobin (g/L) photometrically in whole blood as well as low-haemoglobin dilutions from human and several animal species. The HemoCue system determines the total haemoglobin content (oxy- deoxy- and methaemoglobin but not Sulphaemoglobin).

Actim Fecal Blood (Orion Diagnostica, Oy, Finland) is an immunochromography technique specifically for the determination of human haemoglobin using two monoclonal antibodies. The detection limit in faeces is 50 µg haemoglobin/L or 25–50 µg haemoglobin/g. The method detects the intact human haemoglobin molecule, but neither haemoglobin that is influenced by the enzymes during gastrointestinal passage, nor animal haemoglobin is detectable by this method.

#### *Handling procedures*

*Substudy 1. Intra-assay variation.* Paired measurements of the same faeces samples were made regularly to evaluate the intra-assay variation. Hence 106 paired measurements were made in normal controls (n=30) and patients with gastroenteritis (n=76).

*Substudy 2: Inter-assay variation.* The faecal samples from 8 patients were also analysed in several (2 times n=5, 3 times n=2 and 4 times n=1) consecutive assays in order to calculate the total variation between assays.

*Substudy 3: Freeze-drying.* This substudy comprised 16 faeces samples, 8 from patients with diarrhoea and 8 from healthy volunteers. The samples were diluted 1:6 in distilled water; 1 mL of the suspension was transferred to tubes (Nunc Cryo Tube, Nunc Brand Products, Roskilde, Denmark) and stored at -70°C, and 1 mL was transferred to glass vials (Fiolax clear glass, Pharma Pack, Ingenjörscentrum, Sollentuna, Sweden) and placed at -70°C prior to freeze-drying (Christ Alpha

1–2, Labex, Helsingborg, Sweden). Distilled water (1 mL) was then added to the freeze-dried material prior to analysis.

*Substudy 4: Freeze-thaw cycles.* Eight diarrhoea faeces samples were handled as described above. From faeces samples, 250 µL of the diluted suspension in distilled water (see above) was placed in four separate tubes (Nunc Cryo Tube, Nunc Brand Products, Denmark). The first tube was stored at -70°C immediately. The second tube was freeze-thawed three times (15 min at -70°C followed by 30 min at room temperature). The third tube was freeze-thawed five times, and the fourth tube freeze-thawed ten times. After completion of the freeze-thaw cycles, all of the tubes were stored at -70°C prior to analysis.

*Substudy 5: Time to freeze storage.* Eight diarrhoea faeces samples and eight faeces samples from normal volunteers were handled as described above. The diluted samples in distilled water (250 µL) were placed in 8 separate tubes (Nunc Cryo Tube, Nunc Brand Products, Denmark). The first tube was immediately placed at -70°C. The second to fourth tubes were kept for 6, 12 and 24 h, respectively, at room temperature prior to freeze storage. The fifth to eighth tubes were kept at 6, 12, 24 and 36 h, respectively, at +4°C (refrigerator) prior to storage. All tubes were then stored at -70°C pending analysis.

*Substudy 6: Different buffers.* Faeces samples from 8 patients with diarrhoea were handled as described above. Four different buffers (dilution 1:6): sodium chloride (9 mg/mL, B. Braun Medical AB, Bromma, Sweden), distilled water (B. Braun Medical AB, Bromma, Sweden), phosphate buffer solution (pH 7.4, Apoteket AB, Umeå, Sweden) or formaldehyde 4% buffered (Apoteksbolaget, Gothenburg, Sweden) were added to four samples from each patient; 250 µL of each solution was transferred to tubes (Nunc Cryo Tube, Nunc Brand Products, Denmark) and stored at -70°C prior to analysis.

*Substudy 7: Day-to-day variation.* Faeces samples from 8 healthy volunteers were collected on three consecutive days and stored at

-20°C. The faeces samples were treated as described earlier and diluted 1:6 in distilled water; 250 µL of each solution was transferred to tubes (Nunc Cryo Tube, Nunc Brand Products, Denmark) and stored at -70°C prior to analysis.

*Substudy 8: Fresh handling of faeces.* Fresh faeces samples from 7 healthy volunteers were handled as described above. One fresh sample from each person was diluted in distilled water (1:6) immediately. The rest of the samples were stored at -70°C overnight and were then thawed at room temperature for 30 min and one sample was taken. The sample was diluted in distilled water (1:6); 250 µL of each sample was transferred to tubes and stored at -70°C prior to analysis.

*Substudy 9: Protease inhibitor treating of samples.* The samples from patients (n=8) and healthy controls (n=8) were handled as described above and each sample was divided into two tubes. Protease inhibitor (DMSO solution), a mixture of protease inhibitors with specific inhibition of serine, cysteine, aspartic proteases and aminopeptidases, containing 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin and aprotinin but no metal chelators (protease inhibitor cocktail; Sigma-Aldrich Inc., Saint Louis, MO, USA) in 1% concentration was added to the first tube and both tubes were placed at room temperature for 2 h. They were then stored at -70°C prior to analysis.

*Substudy 10: Length of storage.* In previous studies [14] we determined the concentration of HGF in faeces. In these studies the faeces samples in different volumes were freeze-dried and distilled water was added according to the weight of the samples (1 mL/0.2 g faeces). These prepared samples were then stored at -20°C (February 2001). Available samples (n=32) were now (after 2 years' storage) thawed at room temperature and shaken for 5 min using a Vortex; 1mL faeces dilution was transferred to tubes and stored again at -70°C.

#### *Associations between HGF and albumin and haemoglobin in faeces (substudies 11 and 12)*

The concentration of albumin in faecal samples from healthy volunteers (n=8) and patients with diarrhoea (n=20) was determined. The presence of blood in faeces samples (n=19) was investigated and the concentration of total haemoglobin in faeces samples (n=33) was determined.

#### *Statistics*

The faeces levels of HGF were positively skewed but reasonably well log normally distributed, which is why natural logarithm values were used. All zero values were therefore converted to the lowest detectable level, 0.001. Nevertheless, differences between paired samples were reasonably normally distributed. The methodological intra-assay variation ( $S_{\text{intra}}$ ) was calculated with consecutive paired values using the same kit and expressed as the coefficient of variation ( $\text{COV} = S_{\text{intra}}/\text{mean value}$ , where  $S_{\text{intra}} = \sqrt{(\sum d^2/2n)}$ , where  $d$  = the difference between two consecutive concentrations and  $n$  = the number of paired measurements), first proposed by Dahlberg [16]. The methodological error was expressed in absolute units for values of HGF < 1.0 and by the coefficient of variation for values  $\geq 1.0$ . Since different samples were used in the various substudies, we calculated all the following estimated variations between repeated measurements ( $S_{\text{method}}$ ) including the intra-assay variation. For example, the methodological error of repeated freeze-thaw cycles (assay 4) was estimated by Dahlberg's equation above, which implies that  $S_{\text{method}}$  includes the intra-assay error (sometimes the intra-assay error exceeded the error of the various handling manoeuvres (e.g. the inter-assay variation) and specific errors were therefore not possible to calculate). Differences were analysed by t-test or ANOVA, then later followed by Duncan's test in the case of significance using the logarithm value of HGF. Spearman's non-parametric regression was used in a few instances to investigate possible associations between HGF and other parameters (faecal albumin and haemoglobin). A p-value of less than 0.05 was regarded as statistically significant.

## RESULTS

*Handling procedures*

The methodological variations in the various handling procedures (substudies 1–10) are listed in Table I and are expressed in absolute units for HGF values  $\leq 1$  and in relative units (COV%) for HGF values  $\geq 1$ .

*Substudy 1: Intra-assay variation.* The differences between 106 paired measurements using the same kit are shown in Figure 1. For values of HGF  $\leq 1$  the variation was assessed in absolute units ( $S_{\text{intra}}=0.11$ ;  $n=39$  pairs). For HGF  $\geq 1$  the variation was assessed by the coefficient of variation (COV%;  $S_{\text{intra}}/\text{mean}$  expressed in percent) was 10.5%.

*Substudy 2: Inter-assay variation.* The total coefficient of variation between 4 different assays was, for HGF  $\geq 1$ , 13% ( $S_{\text{method}}=0.59$ ). On the assumption that COV is fairly constant,  $\text{COV}_{\text{inter}} = \sqrt{(\text{COV}_{\text{tot}}^2 - \text{COV}_{\text{intra}}^2)}$ , giving  $\text{COV}_{\text{inter}}=8\%$  for HGF values  $\geq 1.0$ .

*Substudy 3: Freeze-drying.* For HGF values  $\geq 1$ , COV% between paired measurements (freeze-dried as well as non-freeze-

dried) using the same kit was 5.8% and smaller than the intra-assay variation (10.5%, see assay 1). The levels of faeces HGF were significantly higher in the patients with infectious gastroenteritis (non-freeze-dried, median 3.5  $\mu\text{g/L}$ , range 0.2–10.47  $\mu\text{g/L}$ ,  $n=8$ ) compared to the controls (non-freeze-dried, median 0.005  $\mu\text{g/L}$ , range 0–0.03  $\mu\text{g/L}$ ,  $n=8$ ,  $p<0.01$ ).

*Substudy 4: Freeze-thaw cycles.* Faeces HGF levels did not change significantly after several freeze-thaw cycles (3, 5 and 10 times;  $\log(\text{HGF})$ ; repeated measures ANOVA,  $p=0.65$ ) (COV%=4.6% for HGF  $\geq 1$ ; Fig. 2).

*Substudy 5: Time to freeze storage.* Storage for 6, 12 or 24 h at room temperature, or refrigeration for 6, 12, 24 or 36 h (4–6°C) did not affect faeces HGF levels significantly ( $\log(\text{HGF})$ ; repeated measures ANOVA;  $p=0.14$ ) (COV%=11.2% for HGF  $\geq 1$ ; Fig. 3).

*Substudy 6: Different buffers.* Dilution in 4% formaldehyde tended to result in a substantial decrease in HGF (more than 50% reduction in half of the patients). When comparing faecal samples diluted in sodium chloride,

TABLE I. Methodological error of the different substudies.

Substudy	Description	Number of									
		Mean	Obs.	Subjects	Pairs	$S_{\text{method}}$	COV%	Mean	Median	Min.	Max.
1.	Intra-assay variation	HGF < 1	78	39	39	0.11	—	0.16	0.11	0	1.0
		HGF $\geq 1$	134	67	67	2.53	10.5	24.2	6.22	0.6	306
2.	Inter-assay variation	HGF < 1	5	2	3	0.03	—	0.14	0.12	0.1	0.2
		HGF $\geq 1$	35	14	20	0.59	13	4.53	3.52	1.25	10.5
3.	Freeze-drying	HGF < 1	18	9	9	0.06	—	0.16	0.15	0	0.34
		HGF $\geq 1$	14	7	7	0.28	5.8	4.78	3.60	1.74	9.3
4.	Freeze-thaw cycles	HGF < 1	4	1	3	0.04	—	0.10	0.12	0.06	0.13
		HGF $\geq 1$	28	7	21	0.22	4.6	4.73	3.50	1.25	10.7
5.	Time to freeze storage	HGF < 1	8	1	7	0.05	—	0.12	0.12	0.05	0.2
		HGF $\geq 1$	56	7	49	0.50	11.2	4.36	3.28	1.60	10.4
6.	Different buffers	HGF < 1	4	2	2	0.08	—	0.21	0.24	0.05	0.32
		HGF $\geq 1$	12	6	6	0.89	19	4.79	4.84	1.09	8.9
7.	Day-to-day variation	HGF < 1	23	8	15	0.05	—	0.13	0.14	0.03	0.25
		HGF $\geq 1$	—	—	—	—	—	—	—	—	—
8.	Fresh handling of faeces	HGF < 1	14	7	7	0.08	—	0.26	0.27	0.07	0.5
		HGF $\geq 1$	—	—	—	—	—	—	—	—	—
9.	Effects of protease inhibitor	HGF < 1	22	11	11	0.14	—	0.21	0.18	0.05	0.8
		HGF $\geq 1$	8	4	4	1.61	49.7	3.24	2.1	0.55	9.3
10.	Length of storage	HGF < 1	—	—	—	—	—	—	—	—	—
		HGF $\geq 1$	62	31	31	44.5	90	49.5	20.2	0.2	326

HGF=hepatocyte growth factor.

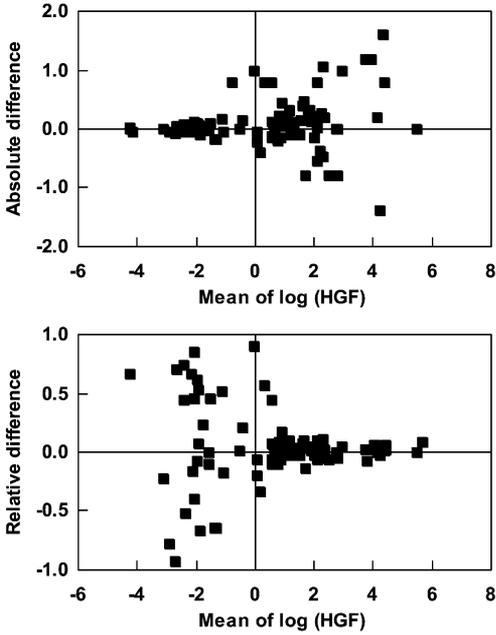


FIG. 1. Differences between paired measurements (HGF1 and HGF2) from the same sample and analysed on the same occasion using the same kit, calculated in absolute ( $\Delta\text{HGF} = \text{HGF2} - \text{HGF1}$ ; upper panels) and in relative units  $\Delta\text{HGF} / (\frac{1}{2}(\text{HGF1} + \text{HGF2}))$ . On the abscissa is the natural logarithm of the geometrical mean, i.e.  $\frac{1}{2}(\log(\text{HGF1}) + \log(\text{HGF2}))$ . HGF = hepatocyte growth factor.

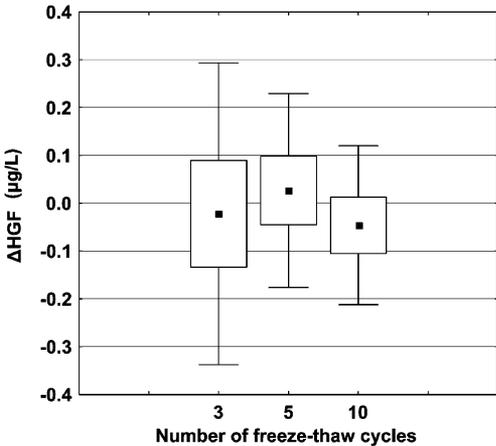


FIG. 2. Differences in faeces hepatocyte growth factor (HGF) levels ( $\Delta\text{HGF}$ ) after 3, 5 and 10 freeze-thaw cycles compared to baseline (0 = thawed once). The differences were not statistically significant. ■: mean; □:  $\pm\text{SE}$ ;  $\perp$ :  $\pm\text{SD}$ .

distilled water or phosphate buffer solution (PBS; pH 7.4) 35% lower values were observed in distilled water compared to PBS or sodium chloride (omitting formaldehyde; log (HGF), repeated measures ANOVA;  $p=0.02$ ;  $n=7 \times 4=28$ ;  $\text{COV}\%=30\%$  for  $\text{HGF} \geq 1$ ). No differences were found when comparing PBS and sodium chloride (Table I) and the  $\text{COV}\%$  was reduced to 19%.

*Substudies 7 and 8: Day-to-day variation and fresh handling of faeces.* There were no day-to-day variations between faeces HGF concentrations in healthy volunteers (log(HGF); repeated measures ANOVA;  $p=0.4$ ;  $n=7 \times 4=28$ ;  $S_{\text{method}}=0.05$ ). There were no significant differences between faeces HGF concentrations in samples that were prepared immediately after defecation and the samples that were initially stored overnight at  $-70^\circ\text{C}$  ( $S_{\text{method}}=0.08$ ).

*Substudy 9: Effect of protease inhibitor.* Treatment of faeces samples with protease inhibitor lowered the HGF levels by around 30% but with a wide variation and the reduction was not statistically significant. Nevertheless, the

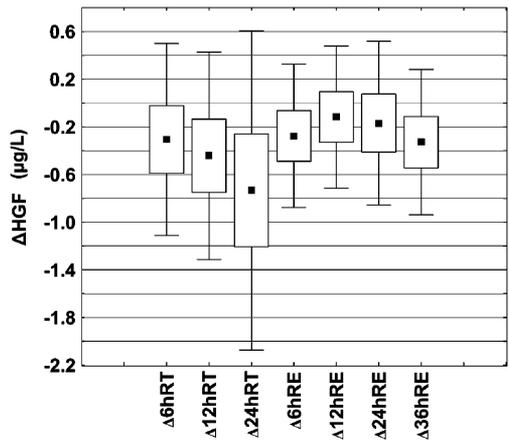


FIG. 3. Differences in faeces hepatocyte growth factor (HGF) levels ( $\Delta\text{HGF}$ ) compared to baseline (0 = separation after one hour at room temperature). The three boxes to the left are faeces kept at room temperature (RT) 6, 12 and 24 h after reconstitution ( $\Delta 6$  h RT,  $\Delta 12$  h RT,  $\Delta 24$  h RT). The four to the right are faeces kept in a refrigerator (RE) (+4–6°C) 6, 12, 24 and 36 h after reconstitution ( $\Delta 6$  h RE,  $\Delta 12$  h RE,  $\Delta 24$  h RE and  $\Delta 36$  h RE). The differences were not significant. ■: mean; □:  $\pm\text{SE}$ ;  $\perp$ :  $\pm\text{SD}$ .

methodological error was roughly tripled (Table I) giving an absolute  $S_{\text{method}}=0.14$  ( $\text{HGF} < 1$ ) and  $\text{COV}\%=50\%$  ( $\text{HGF} \geq 1$ ).

*Substudy 10: Length of storage.* The levels of faeces HGF obtained by ELISA in January 2003 (mean 45.7, median 16.5, range 0.2–234 ng/g HGF) were slightly, but significantly lower than the values obtained by ELISA in the same samples in February 2001 (mean 53.3, median 30, range 1.18–326 ng/g HGF) (Mean of  $\log(\text{HGF } 2001)=2.93$  and in 2003 2.55; Student's t-test,  $p=0.03$ , not significant using a non-parametric test,  $p=0.6$ ). There was, however, a considerable variation in both directions and the calculated  $S_{\text{method}}$  was virtually unaffected by using the predicted 2001 value (from the regression analysis on the log values followed by antilogarithm) ( $\text{COV}\%=90$  for  $\text{HGF} \geq 1$  using measured values and  $\text{COV}\%=83\%$  when using the predicted value).

*Associations between HGF and albumin and haemoglobin in faeces (substudies 11 and 12)*

There was no significant correlation between faeces HGF levels and faeces albumin levels in the same sample from patients with infectious gastroenteritis (Spearman rank correlation,  $r=0.09$ ,  $p=0.7$ ;  $n=20$ ). The albumin level in normal faeces was below the detection limit of the assay ( $<6$  mg/L) in all cases (substudy 11). None of the healthy controls had blood in their faeces (substudy 12). In four patients with infectious gastroenteritis, the presence of blood in faeces was observed (Actim Fecal Haemoglobin) (a few observations not allowing a statistical comparison). There was a significant negative correlation between the total haemoglobin (HemoCue) and faeces HGF levels ( $n=33$ ,  $r=-0.54$ ,  $p<0.05$ ). In patients with infectious diarrhoea, the correlation coefficient ( $r$ ) was  $-0.52$  ( $p=0.07$ ,  $n=12$ ) and the corresponding value for the patients with normal faeces was  $-0.20$  ( $p=0.43$ ,  $n=21$ ). There was no correlation between the presence of blood in the faeces (Actim Fecal Haemoglobin) and total haemoglobin levels (HemoCue) ( $p=0.77$ ).

## DISCUSSION

In this work the stability of HGF in faeces samples was assessed for the first time. We showed that various mechanical and chemical strains relevant to sample handling do not significantly affect the faecal HGF concentration. Although a number of groups have studied the concentration of cytokines such as IL-6 and TNF- $\alpha$  in stool samples [17, 18], as far as we know, the presence and stability of HGF in faeces has not been investigated before.

Recently, we studied the stability of HGF determination in serum samples [15] and found that HGF was stable in serum samples.

The faeces HGF level was very low in normal controls and therefore the variation in different conditions is not considered reliable in these cases. We therefore also studied faeces from patients with infectious diarrhoea that had higher levels of HGF [14]. We observed that storage at different temperatures and different periods of storage, several freeze-thaw cycles, freeze-drying and the buffers used (other than formaldehyde) did not significantly influence the results. However, using 4% formaldehyde resulted in decreased HGF levels at concentrations  $\geq 2.0$   $\mu\text{g/L}$ , which is in agreement with the known property of this substance, i.e. denaturation of proteins. The HGF concentration was not influenced by processing the faecal samples with protease inhibitor. Although we did not investigate the stability of HGF at a molecular level, and the HGF concentrations were determined by ELISA, which measures both the single-chain HGF precursor and the double-chain active HGF [19], we speculate that the structure of HGF is stable despite the presence of constituent elements of faeces.

Ferguson *et al.* (1995) [20] determined the concentrations of various substances (IgG, IgM, IgA, albumin, alpha-1 antitrypsin and haemoglobin) in whole-gut lavage fluid (WGLF) and faeces from patients with inflammatory bowel diseases and in healthy controls. Using both protease inhibitor-processed and unprocessed samples, these investigators observed differences in the concentration of substances in faeces and WGLF. They suggest that immunological tests on faecal extracts might be misleading, considering determination in WGLF as a more appropriate marker for bowel integrity. They discuss a number of reasons for this

phenomenon, such as instability of substances in faeces caused by digestive enzymes and bacterial proteases, variations in faecal water content as well as leakage of substances derived from plasma into the gut lumen over an inflamed mucosa. However, they found that when haemoglobin was assayed by a method which detects bacterial degradation products as well as the native substance (HemoQuant), more than 85% of ingested haemoglobin could be measured in both faeces and WGLF. Thus, based on the stable structure of haemoglobin, they did calculations expressing the concentration of various proteins per milligram of haemoglobin.

The amount of albumin in faeces might indicate a leakage from blood to faeces during inflammation [20]. Choudari *et al.* [21] have shown that the levels of albumin increased in GLF during inflammatory bowel disease. We determined the values of albumin in the faeces samples and found no correlation between HGF and albumin levels even after protease inhibitor processing of the samples (data not shown). This finding supports our assumption that a local production of HGF might be the case during acute gastroenteritis, which is reflected in high levels of HGF in faeces [14]. This is in line with our previous observations of local production of HGF during inflammation in bacterial meningitis (cerebrospinal fluid) [13] and pneumonia (exhaled breathe condensate) [22].

We studied the faeces haemoglobin by two methods; HemoCue for total haemoglobin and Actim Fecal Blood for detection of human haemoglobin in faeces. We observed that healthy controls had low HGF concentration, normal faeces consistence and high levels of total haemoglobin (HemoCue). The patients with infectious diarrhoea had high faeces HGF, loose faeces consistence and very low total haemoglobin. We also observed that patients with non-infectious diarrhoea have low faeces HGF, loose faeces consistence and low total faeces haemoglobin (unpublished observation). These findings indicate the influence of water content on the total haemoglobin concentration in faeces samples, and are in agreement with the observations of Ferguson *et al.* [20]. Determination of total faecal haemoglobin might thus be a method to control for water content.

Although we previously observed that there

was no correlation between HGF levels in faeces and serum, serum and faeces HGF levels that were increased in the acute stage of infectious gastroenteritis decreased during the course of disease and normalized at convalescence [14]. However, there were no day-to-day variations in values of HGF in serum [15] and faeces (assay 7) of healthy controls.

We conclude that HGF is stable in faeces and determination of faecal HGF with ELISA is a reliable method that could be used in future studies. We recommend the following procedure, which gave reliable results during various mechanical and chemical strains: fresh stool is reconstituted in distilled water and is centrifuged at 1000 g in 10–15 min, the supernatant is transferred to tubes and stored at ~70°C before analysis. This preparation can be stored for at least 6 months.

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