The effects of hydroxycarbamide on the plasma proteome of children with sickle cell anaemia

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Summary

We have investigated changes in the plasma proteome of children with sickle cell anaemia (SCA) associated with hydroxycarbamide (HC) use, to further characterize the actions of HC.

51 children with SCA consented to take part in this study. 18 were taking HC at a median dose of 22mg/kg, and 33 were not on HC. Plasma was analyzed using an unbiased proteomic approach and a panel of 92 neurological biomarkers. HC was associated with increased haemoglobin (Hb) (89.8g/l vs 81.4g/l, P=0.007) and HbF (15.3 vs 6.7%, P<0.001). 17 proteins were decreased on HC compared to controls by a factor of <0.77, and six proteins showed >1.3 fold increased concentration. HC use was associated with reduced haemolysis (lower  globin chains, haptoglobin related protein, complement C9; higher hemopexin), reduced inflammation (lower -1-acid glycoprotein, CD5 antigen-like protein, ceruloplasmin, factor XII, immunoglobulins, cysteine-rich secretory protein 3, vitamin D-binding protein) and decreased activation of coagulation (lower factor XII, carboxypeptidase B2, platelet basic protein). There was a significant correlation between the increase in HbF% on HC and hemopexin levels (R=0.603, P=0.023). This study demonstrated three ways in which HC may be beneficial in SCA, and identified novel proteins, which may be useful to monitor therapeutic response.

Introduction

The most prevalent and severe type of sickle cell disease is caused by homozygosity for the sickle mutation (HBB; c.20A>T, p.Glu7Val), and referred to as sickle cell anaemia (SCA). It is one of the commonest severe inherited disorders in the world, and associated with unpredictable acute complications, progressive organ damage and shortened life expectancy(Brousse*, et al* 2014). Although outcomes have improved significantly in high-income countries over the last 40 years, there are still very few treatments available, with HC continuing to be the only drug which has convincingly been shown to modify the natural history of the condition and potentially prevent organ damage(McGann and Ware 2015).

Randomized controlled trials have shown that HC reduces the frequency of acute pain and acute chest syndrome(Charache*, et al* 1995, Wang*, et al* 2011), reduces the need for blood transfusion(Charache*, et al* 1995), reduces the risk of infection and malaria in some settings(Tshilolo*, et al* 2018), and is effective as part of the management of abnormal transcranial Doppler (TCD) velocities(Ware*, et al* 2016). Uncontrolled studies also suggest that HC increases oxygen saturations(Singh*, et al* 2008), decreases albuminuria(Tehseen*, et al* 2017) and may increase life expectancy in adults(Steinberg*, et al* 2003). In the USA and UK it is recommended that HC is offered to all children with SCA (Qureshi*, et al* 2018, Yawn*, et al* 2014), and approximately 80% children take it in some large centres in the USA; in Europe practice varies widely, but HC is offered to most symptomatic children.

The main mechanism of action of HC is to increase haemoglobin F (HbF) production, which decreases the rate of HbS polymerization, with subsequent improvement in many downstream pathologies, including reductions in vaso-occlusion, inflammation, anaemia and haemolysis(Ware 2010). HC has other actions beyond the  globin locus, including reducing white cell numbers, decreasing expression of adhesion molecules on the vascular endothelium and possibly improving nitric oxide metabolism(Rees 2011), although it is unclear how much these contribute to its therapeutic actions. In a mouse model of sickle cell disease in which HC caused no increase in HbF levels, there was no evidence of improvement in clinical or laboratory parameters(Lebensburger*, et al* 2010).

Another important unanswered question involves variability in the therapeutic response to HC. It is unclear why some patients appear not to respond as well as others. Some of this may be related to drug adherence, although all studies show a variable response. In the HUSTLE study the HbF level varied from 16.2 – 27.8% after one year in patients achieving the maximum tolerated dose(Estepp*, et al* 2017); some of this variability is related to baseline HbF levels, although analysis of data from the HUG-KIDS study showed that this explained only about 18% of the HbF response(Ware*, et al* 2002).

Previous studies have measured the effects of HC on selected biomarkers, such as indicators of inflammation (Penkert*, et al* 2018) or vasculopathy (Lapoumeroulie*, et al* 2005), but we have adopted an unbiased proteomic approach to identify changes in plasma protein levels associated with HC use. We hypothesized that there would be significant differences in the plasma proteome between children with SCD taking and not taking HC, and that these differences would shed light on the mechanism of action of HC and may identify novel biomarkers associated with response to HC.

Methods

*Patients and setting*

The data were collected as part of a study of silent cerebral infarcts (SCIs), funded by the Stroke Association (Grant TSA 2012/06)(Tewari*, et al* 2018). The study was approved by the UK National Research Ethics Committee (reference 13/LO/0709) and all patients/parents gave written consent. Children with SCA were recruited from clinics at King’s College Hospital and the Evelina Children’s Hospital. The aim was to recruit 50 patients, with approximately equal numbers of those with SCIs and controls with normal brain MRIs. Children already known to have SCIs from previous MRI scans were selectively recruited and eligible patients without previous MRIs were recruited sequentially as they attended clinic. Children taking HC were recruited to both arms and had been on a stable dose of HC for at least 6 months. Inclusion criteria were: sickle cell anaemia (HbSS), age 8-18 years old, normal or conditional TCD velocities (<200cm/s), able to tolerate MRI scan without sedation. Exclusion criteria were: history of overt stroke, blood transfusion in last 4 months, serious co-existing disorder, acute complications requiring hospital attendance in last 3 months.

*Routine Clinical and Laboratory data*

Routine steady-state laboratory analyses were recorded, including haemoglobin, haemoglobin F levels, white cell count, reticulocyte count, renal function, hepatic function, lactate dehydrogenase, G6PD status, and C-reactive protein.

*Proteomic Analysis*

Blood samples were collected on all children at the time of consent. A data-driven proteomics approach was used to discover protein changes in the plasma associated with SCI. We used an established a workflow combining isobaric Tandem Mass Tags (TMT) reagents with 1D gel separation, followed by high sensitivity rapid throughput MS and MS/MS analysis, referred to as TMT-GelC/MS/MS. TMT®s (Proteome Sciences plc) are a set of isobaric mass tags for labeling proteins and peptides at amine functions and subsequent mixing of up to six different protein samples. During MS/MS, each set of tags gives rise to different reporter ions with specific molecular masses thus allowing them to share the same chemical structure but have different molecular weights dependent on the different number of heavy isotopes contained. Peptides labelled with different TMTs show identical chromatographic retention times as well as identical ionization and fragmentation behaviour of tags and tagged species.

The TMT-GeLC-MS/MS method involved TMT labelling of samples, pooling and then single 1D electrophoresis. Fractionation by gel sectioning (~10 sections) was followed by in-gel digestion prior to LC/MS/MS. This method of labelling proteins, rather than labelling digested peptides, reduced variation in reporter intensities from bias introduced by loading artefacts and extraction efficiency. The use of the initial 1D gel allowed separate analysis of gel regions containing more protein from those containing fewer proteins leading to more even coverage of the proteome.

Protein discovery study was performed in a reference-controlled manner using the TMT-GeLC/MS/MS workflow and this approach generated considerable coverage

of the plasma proteome(Engmann*, et al* 2010).

Mass spectral data were acquired using an Orbitrap Velos mass spectrometer (Thermo Scientific). Compilation and validation of the peptide and protein assignments were carried out using Proteome Discoverer v1.3 (Thermo) and Scaffold v2.6 (Proteome Software Inc) mass spectrometry software. Proteins were quantified based on the intensities of the reporter ions released from the TMT tags during MS/MS of peptides. The use of the TMT6plex reagents enabled the simultaneous analysis of a five samples alongside an overall study reference comprising of a pool of all subjects. The proteomics study comprised 10 separate TMT6plex experiments, each with 5 samples and a reference channel for normalization purposes. 10 gel sections from each TMT set were analysed by LC/MS/MS.

Data were exported for each TMT set into an Excel file, and duplicate peptides were removed. The data were filtered so that only peptides identified in all 10 sample channels were analysed further. The data were normalized and merged against the peptide sequence using R software. The merged data were further filtered such that only peptides present in all patient samples were studied. Relative amounts of each peptide were compared between the SCI and control groups, and P values calculated for 1.3 fold differences.

*Targeted plasma analysis for biomarkers of neurological disease*

Plasma samples were also analysed for 92 protein biomarkers known to be implicated in neurological disease, using a multiplexed proximity extension assay(Assarsson*, et al* 2014). Plasma samples, anticoagulated with EDTA, were frozen at -80oC within 6 hours of venesection, and transported as one batch to Olink Proteomics in Sweden (www.olink.com/proseek-multiplex) on dry ice for analysis using their Proseek Multiplex Neurology I panel (see Supplementary Materials for full list of biomarkers).

*Data Analysis*

Means were compared with adjustments for multiple comparisons when appropriate. Statistical analyses were performed in the Proteomics Laboratory and medical statistics department of Public Health Sciences at King’s College London.

Results

*Basic laboratory measurements*

51 patients (22 female) were included in the study, and 18 of these were taking HC. The median dose of HC was 22 (range 18 – 24) mg/kg/day. 19 had SCIs and 32 normal brain MRI. There was no significant difference in HC use between SCI and control groups: 7/19 children with SCIs were taking HC compared to 11/32 without SCIs (Chi square, P=0.859)(Tewari*, et al* 2018). G6PD status was known for 45 patients and there was no association with HC use; G6PD deficiency was present in 2/16 on HC and 3/29 controls (Chi square, P=0.826). Steady-state laboratory data are given in Table 1. These measurements showed the expected patterns, and in particular the significant increase in HbF levels associated with HC use (6.7 vs 15.3%, P<0.001).

*Plasma proteomic and targeted analysis*

4662 different peptides were identified across all patients. Of these 1312, from 346 different proteins, were present in all 51 patients and were used for ongoing analysis. Peptides from the fibrinogen gamma chain and Complement C3 showed inconsistent patterns, with some peptides elevated on HC and others decreased, and were not considered further.

On unbiased proteomic analysis, six proteins were present at significantly increased concentrations in children on HC (Table 2), and 17 were significantly lower with HC (Table 3), including alpha, beta and delta globin chains.

Targeted analysis of the samples for biomarkers of neurological disease showed significant differences in 18 proteins between those taking and not taking HC, but only three of these remained significant after correction for multiple comparisons (Table 4).

*Correlation between changes in HbF levels in HC and plasma proteins*

Changes in HbF% on HC were calculated by subtracting the HbF% immediately before starting HC from the HbF% on the day when the sample for proteomic analysis was taken. Correlation coefficients were calculated between these changes in HbF% and concentrations of proteins significantly changed in those on hydroxyurea. The change in HbF% correlated significantly with plasma concentrations of hemopexin (R=0.603, P=0.023), ceruloplasmin (R=-0.589, P=0.023), immunoglobulin J chain (R=0.537, P=0.048) and platelet basic protein (R=-0.581, P=0.029) (Table 5). For patients taking HC, correlation coefficients were also calculated between HbF% on the day of consent and protein levels: HbF% correlated significantly with hemopexin (R=0.503, P=0.013), and also and globin levels, all of which suggest that greater increases in HbF% on hydroxyurea are associated with decreased rates of intravascular haemolysis. No such relationship was seen on analysis of the pre-HC HbF% (Table 5).

Discussion

Routine laboratory measurements showed the expected changes, with HC associated with higher HbF and haemoglobin levels, reduced reticulocytes, increased MCV and reduced neutrophil counts, all of which may be of some therapeutic value, as well as being indirect indicators of reduced HbS polymerization.

Peptides from six proteins were found at >1.3 fold higher concentrations in children taking HC. It is difficult to know the significance of increased levels of -1B-glycoprotein and -2-macroglobulin: -1B-glycoprotein is an immunoglobulin-like protein of unknown function and -2-macroglobulin is a multi-functional proteinase inhibitor, which has been found at increased concentrations in patients with SCA(Makis*, et al* 2000). Apolipoprotein C-III is central to triglyceride metabolism(Norata*, et al* 2015) but again the biological significance of elevated levels in patients with SCA on HC is unclear. In SCA, plasma fibronectin has been implicated in the adherence of reticulocytes to monocytes(Brittain*, et al* 2008), and elevated levels associated with HC could potentially be detrimental, although it could also reflect reduced consumption associated with lower reticulocyte and monocyte counts. Similarly, plasma vitronectin levels are increased in children taking HC, and this glycopeptide is implicated in intercellular adhesion and control of the extracellular environment(Leavesley*, et al* 2013). These findings require further investigation. Perhaps most interestingly, increased hemopexin concentrations were also found in association with HC; hemopexin is the scavenger molecular for free haem, and levels have previously been shown to be depleted in SCA(Santiago*, et al* 2018), with some evidence of therapeutic benefit from hemopexin infusion(Belcher*, et al* 2018). Our finding of increased hemopexin levels associated with HC has not been shown before, and is in keeping with decreased rates of haemolysis, and suggests another probably beneficial action of HC in SCA.

Proteomic analysis identified peptides from 17 different proteins which were found at >1.3 fold higher concentrations in controls. As for hemopexin, some of these are attributable directly to the reduced rate of red cell destruction associated with higher HbF levels:   globin chains and haptoglobin-related protein; the latter binds haemoglobin but is not removed by CD163, and is thought to be included in measurements of free plasma haemoglobin(Nielsen*, et al* 2006). Complement C9 has been shown to bind to denser sickle cells and increase susceptibility to lysis, and lower levels with HC may be either a cause or effect of reduced haemolysis(Test and Woolworth 1994). The reduced levels of all of these are in keeping with lower levels of plasma haemoglobin, which is thought to be a significant causal factor in the endothelial dysfunction which is characteristic of SCA(Reiter*, et al* 2002).

A second group of proteins found at lower levels in those on HC were markers of inflammation, suggesting that HC is associated with decreased inflammation in SCA: -1-acid glycoprotein (also called orosomucoid) for which high urinary levels are associated with kidney disease in SCA(Jerebtsova*, et al* 2018), CD5 antigen-like protein(Prentice*, et al* 2010), ceruloplasmin, factor XII, immunoglobulins ( chain C region, and J chain), vitamin D-binding protein(Chishimba*, et al* 2010) and cysteine-rich secretory protein 3, which is released on neutrophil degranulation(Udby*, et al* 2002).

A third group of proteins present at lower levels in those on HC involved procoagulant factors: Factor XII; carboxypeptidase B2, which attenuates fibrinolysis(Leenaerts*, et al* 2018) and platelet basic protein, which is released by activated platelets(El-Gedaily*, et al* 2004). It is harder to interpret the decreased levels of serotransferrin in those taking HC.

We assessed the potential clinical significance of the plasma protein differences by calculating correlation coefficients between plasma proteins and changes in HbF% associated with HC use. Markers of intravascular haemolysis (hemopexin, globin chains) correlated positively with HbF% at the time of blood sampling, and hemopexin also correlated also with the increase in HbF% associated with HC use, confirming that higher HbF levels are associated with reduced intravascular haemolysis. Interestingly, there was no correlation between inflammatory markers and HbF% on HC, although there was a negative correlation between the increase in HbF% with HC and two inflammatory markers (ceruloplasmin and platelet basic protein). This possibly suggests that children with less inflammation respond with a bigger increase in HbF% on HC, although this needs to be studied in larger, prospective studies.

Three of the targeted panel of neurological biomarkers were significantly reduced in those taking HC. Plexin-B3 is expressed in brain tissue and involved in axon guidance(Rujescu*, et al* 2007), and possibly lower levels suggest reduced neuronal damage associated with HC use. Reduced interleukin 12 and leukocyte-associated immunoglobuin-like receptor-2(Lebbink*, et al* 2008) levels both suggested reduced inflammation associated with HC, in keeping with the findings of the unbiased proteomic analysis.

Our study is cross-sectional rather than longitudinal, raising the possibility that the differences between the two groups could be related to pre-existing clinical differences rather than as a consequence of HC use. This is unlikely in that the changes in the HC group (higher haemoglobin, less inflammation etc) indicate less severe disease, whereas in general more severely affected children are offered HC.

Using an unbiased proteomic approach, our study identifies plasma proteins that are modulated by HC use and are associated with pathways involved in decreased rates of haemolysis (6 proteins), reduced inflammation (9 proteins), and reduced hypercoagulability (3 proteins). Such differences are largely explicable as downstream changes associated with higher HbF levels and slower HbS polymerization(Lebensburger*, et al* 2010), resulting in less red cell damage and vaso-occlusion, and are in keeping with the previously documented therapeutic actions of HC(Ware 2010). Although these results need confirming in prospective longitudinal studies, some or all of the proteins identified in our study may be useful to monitor patients treated with HC, potentially to optimize and individualize the dose used.

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JB analysed the data and contributed to writing the manuscript. ST recruited patients, prepared samples and contributed to writing the manuscript. SM helped analyse data and contributed to writing the manuscript. FK helped design the study and contributed to writing the manuscript. BI recruited patients to the study and contributed to writing the manuscript. GR performed proteomic studies, analysed the data and contributed to writing the manuscript. MW helped design the study, supervised proteomic studies, helped analyse the data and contributed to writing the manuscript. DR designed the study, analysed the data and wrote the manuscript.

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|  |  |  |  |
| --- | --- | --- | --- |
|  | Controls | Hydroxyurea | P value |
|  | n | mean | s.d. | n | mean | s.d. |  |
| age (years) | 33 | 11.6 | 3.83 | 18 | 12.4 | 2.50 | 0.34 |
| **Hb (g/l)** | **33** | **81.4** | **7.29** | **18** | **89.8** | **10.85** | **0.007** |
| **MCV (fl)** | **33** | **83.2** | **7.43** | **18** | **94.3** | **13.70** | **0.004** |
| **HbF (%)** | **33** | **6.7** | **4.14** | **18** | **15.3** | **8.02** | **<0.001** |
| **HbF (g/l)** | **33** | **5.5** | **3.6** | **18** | **14.1** | **8.18** | **<0.001** |
| **neutrophils (x109/l)** | **33** | **5.0** | **1.50** | **18** | **3.8** | **1.81** | **0.017** |
| **Reticulocytes****(x109/l)** | **33** | **389** | **92.2** | **18** | **227** | **89.1** | **<0.001** |
| bilirubin | 33 | 42.7 | 16.3 | 18 | 38.9 | 19.4 | 0.46 |
| AST | 33 | 59.8 | 24.5 | 18 | 51.7 | 21.9 | 0.25 |
| **LDH** | **32** | **601** | **141** | **18** | **505** | **113** | **0.017** |

Table 1: Comparison of laboratory and clinical measurements of those taking HC with those who were not. P values from t-tests, with significant results shown in bold (P<0.05).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Protein** | **Number different peptides** | **Normalized concentration in controls** | **Normalized concentration on HU** | **Fold change on HU** | **P value** |
| Alpha-1B-glycoprotein | 2 | 0.392 | 0.640 | 1.63 | 0.013 |
| Alpha-2-macroglobulin | 71 | 0.895 | 1.17 | 1.31 | 0.003 |
| Apolipoprotein C-III | 2 | 1.005 | 1.4 | 1.39 | 0.0039 |
| Fibronectin | 17 | 0.729 | 0.968 | 1.33 | 0.00001 |
| Vitronectin | 1 | 1.115 | 1.93 | 1.7 | 0.007 |
| Hemopexin | 11 | 0.912 | 1.405 | 1.54 | 0.00098 |

Table 2: Alphabetical list of all proteins with peptides at significantly increased concentrations (>1.3 fold) in children taking HC compared to controls. Where more than one peptide was increased from the same protein, the most significant difference is given. Concentrations are normalized against standard controls.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Protein** | **Number different peptides** | **Normalized concentration in controls** | **Normalized concentration on HU** | **Fold change on HU** | **P value** |
| Alpha-1-acid glycoprotein 1 | 2 | 0.619 | 0.412 | 0.66 | 0.001 |
| Carboxypeptidase B2 | 1 | 0.702 | 0.526 | 0.75 | 0.012 |
| CD5 antigen-like | 3 | 1.125 | 0.826 | 0.73 | 0.004 |
| Ceruloplasmin | 40 | 1.23 | 0.873 | 0.71 | 0.001 |
| Coagulation factor XII | 1 | 0.887 | 0.676 | 0.76 | 0.011 |
| Complement component C9 | 1 | 0.840 | 0.642 | 0.76 | 0.001 |
| Complement factor B | 1 | 1.43 | 1.015 | 0.71 | 0.014 |
| Cysteine-rich secretory protein 3 | 1 | 0.594 | 0.451 | 0.76 | 0.0007 |
| Haptoglobin-related protein | 1 | 2.21 | 1.385 | 0.63 | 0.007 |
| Hemoglobin subunit alpha | 95 | 2.065 | 1.28 | 0.62 | 0.002 |
| Hemoglobin subunit beta | 24 | 1.395 | 0.856 | 0.61 | 0.003 |
| Hemoglobin subunit delta | 2 | 2.42 | 1.35 | 0.56 | 0.0006 |
| Immunoglobulin mu chain C region | 1 | 1.87 | 1.38 | 0.74 | 0.04 |
| Immunoglobulin J chain | 1 | 1.095 | 0.801 | 0.73 | 0.02 |
| Platelet basic protein | 7 | 0.937 | 0.53 | 0.57 | 0.002 |
| Serotransferrin | 9 | 1.47 | 1.085 | 0.74 | 0.002 |
| Vitamin D binding protein | 2 | 2.95 | 1.84 | 0.62 | 0.005 |

Table 3: Alphabetical list of all peptides and proteins with concentrations decreased by a factor of more than 0.77 in children taking hydroxyurea compared to controls. Where more than one peptide was identified, the most significant difference is given. Concentrations are normalized against standard controls.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Hydroxyurea** | **Controls** | P |
|  | N | mean | N | mean |  |
| PLXNB3 | 20 | 19.7 | 32 | 26.9 | 0.03 |
| IL-12 | 20 | 200 | 32 | 329 | 0.03 |
| LAIR-2 | 20 | 12.2 | 32 | 17.9 | 0.03 |

Table 4: Concentrations of neurological biomarkers in children with sickle cell anemia, which were significantly different between patients on hydroxyurea and controls, after correction for multiple comparisons. Figures are based on normalized protein expression, assessing relative protein quantitation. PLXBN3 = plexin-B3, IL-12 = interleukin 12 (measured as IL-12A and IL-12B subunits), LAIR-2 = leukocyte-associated immunoglobulin-like receptor 2.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Pre-HC HbF% | HbF% on HC | HbF% change on HC |
| n | R | P | n | R | P | n | R | P |
| Alpha-1B-glycoprotein | 14 | .020 | .945 | 21 | .370 | .099 | 14 | .294 | .308 |
| Alpha-2-macroglobulin | 14 | .271 | .348 | 21 | .288 | .206 | 14 | -.053 | .858 |
| Apolipoprotein C-III | 14 | -.353 | .216 | 21 | .217 | .346 | 14 | .114 | .698 |
| Fibronectin | 14 | .307 | .285 | 21 | .332 | .142 | 14 | -.102 | .730 |
| Vitronectin | 14 | -.021 | .944 | 21 | .259 | .257 | 14 | .064 | .829 |
| Hemopexin | 14 | -.208 | .476 | 21 | **.533** | **.013\*** | **14** | **.603** | **.023\*** |
| Alpha-1-acid glycoprotein 1 | 14 | .153 | .601 | 21 | -.277 | .224 | 14 | -.471 | .089 |
| Carboxypeptidase B2 | 14 | .494 | .073 | 21 | .322 | .155 | 14 | .109 | .710 |
| CD5 antigen-like | 14 | -.157 | .592 | 21 | -.296 | .193 | 14 | .151 | .605 |
| Ceruloplasmin | 14 | .322 | .262 | 21 | -.406 | .068 | **14** | **-.589** | **.027\*** |
| Coagulation factor XII | 14 | -.437 | .119 | 21 | .122 | .599 | 14 | .187 | .522 |
| Complement component C9 | **14** | **.631** | **.015\*** | 21 | .375 | .094 | 14 | .341 | .233 |
| Complement factor B | 14 | -.175 | .549 | 21 | -.017 | .942 | 14 | -.019 | .948 |
| Cysteine-rich secretory protein 3 | 14 | .401 | .155 | 21 | -.366 | .103 | 14 | -.290 | .315 |
| Haptoglobin-related protein | 14 | -.315 | .272 | 21 | -.241 | .292 | 14 | .290 | .315 |
| Hemoglobin subunit alpha | 14 | .119 | .685 | 21 | **-.514** | **.017\*** | 14 | -.412 | .143 |
| Hemoglobin subunit beta | 14 | .211 | .470 | 21 | **-.515** | **.017\*** | 14 | -.382 | .178 |
| Hemoglobin subunit delta | 14 | -.016 | .958 | 21 | **-.571** | **.007\*** | 14 | -.467 | .092 |
| Immunoglobulin mu chain C region | 14 | -.140 | .634 | 21 | -.250 | .275 | 14 | -.008 | .978 |
| Immunoglobulin J chain | 14 | .231 | .427 | 21 | .181 | .431 | **14** | **.537** | **.048\*** |
| Platelet basic protein | 14 | .210 | .417 | 21 | -.372 | .097 | **14** | **-.581** | **.029\*** |
| Serotransferrin | 14 | .184 | .528 | 21 | .095 | .683 | 14 | .312 | .278 |
| Vitamin D binding protein | 14 | -.065 | .825 | 21 | -.125 | .589 | 14 | .256 | .377 |

Table 5: Pearson correlation coefficients between plasma proteins (at the time of entry into the study) and HbF percentage before starting hydroxycarbamide, when taking hydroxyurea (at time of entry into the study) and the increase in HbF percentage on hydroxycarbamide. 2-tailed P values are give and P values <0.05 considered significant (marked with \* and in bold).