GDF -15 AND SEVERITY SCORES IN SICKLE CELL DISEASE PATIENTS ATTENDING NNAMDI AZIKIWE UNIVERSITY TEACHING HOSPITAL, NNEWI, ANAMBRA STATE, NIGERIA

ABSTRACT

AIM

Granulocyte differentiation factor 15 (GDF15) is a growth factor and biomarker for many disorders where ischaemia reperfusion injury (IRI) is pathophysiologically relevant. Hence the need to evaluate GDF-15 as a biomarker in sickle cell disease (SCD).

STUDY DESIGN

This is a cross sectional study.

PLACE AND DURATION OF STUDY

Department of Haematology, Nnamdi University Teaching Hospital, Nnewi, Anambra state, Nigeria, between January and December 2018

METHOD

Ninety subjects were randomly recruited with haemoglobin (Hb) phenotypes SS (test), AS and AA (controls); numbering 30, 28 and 32 respectively. Disease severity was determined by calculating an objective score. 5 mls of blood was collected and used to determine Full Blood

Count (FBC), haemoglobin Phenotype and GDF-15 levels (by Enzyme Linked Immunosobent assay) . Data collected was analysed using Statistical Package for Social Sciences software version 20 (SPSS Inc., IL, Chicago, USA). P> 0.05 was considered as significant.

RESULT

GDF-15 level was found to be significantly different in the different HB phenotypes p= 0.005 and correlated negatively with sickle cell disease severity (r= -0.307, p= 0.098). The difference between median GDF-15 levels of HBSS subjects with mild and moderate disease was statistically significant at p= 0.01

CONCLUSION

We hypothesize that GDF-15 may be a potential therapeutic target for intervention against ischaemia/reperfusioninduced micro- vascular injury. Polyphenols in Cassava [Manihot esculenta Crantz (MEC)] leaves may be useful in taking advantage of this potential therapeutic target.

Key words: GDF- 15, Sickle cell disease, Ischaemia reperfusion injury, Polyphenols, Manihot esculenta Crantz leaves

Research in context

The standard of care and available drugs to definitely treat sickle cell disease (SCD), does not include drugs that address ischaemia reperfusion injury (IRI), which is a very important part of the SCD pathophysiology.

Added value of this study: Our study demonstrates that GDF-15 may be a potential therapeutic target for intervention against IRI induced micro-vascular injury.

Implications of all the available evidence: It is potentially possible to use Polyphenols found in Cassava (Manihot esculenta Crantz) leaves which function similarly as therapy for SCD.

Introduction

Haemoglobinopathies are the single most important genetic disorder in the world.[1] In Nigeria alone 2-4% and 25-30% of the population suffer from sickle cell disease (SCD), and carry the sickle cell gene respectively.[2] In sub-Saharan Africa, where there is a high burden of this disease, malaria, which maintains and drives this gene in the population due to balanced disequlibrium, is still endemic.[3] This seems to tip the equilibrium towards an increase in prevalence.

This disorder is caused by a mutation that causes glutamine to be replaced by valine in the 6th position of the beta haemoglobin chain, causing haemoglobin molecules to polymerize when they are deoxygenated. An extension of this pathophysiological process brings about the protean manifestations of SCD in every tissue and system of the body. Though SCD patients have exactly the same genetic mutation, they present with a wide range of phenotypes; the reason why this happens is not completely understood. Finding biomarkers that predict these phenotypes has become very important for individualized therapy[4,5,6], some of these biomarkers may also become useful as therapeutic tools.

Granulocyte differentiation factor 15 (GDF15) is a growth factor that is part of the bone Morphologic protein (BMP) group of genes and is produced in the CNS and macrophages under physiological conditions and has been found to be an important biomarker for many disorders,

including those where the pathophysiological process involves injury caused by ischaemia and reperfusion (IRI)[7,8] IRI is a very important event in the pathophysiology of SCD[9], hence it becomes important to evaluate GDF15 as a biomarker in SCD patients.

Materials and Methods

A total of ninety subjects were randomly recruited from sickle cell disease clinics in Nnamdi Azikiwe university teaching hospital in Anambra State, Nigeria. Thirty were homozygous SCD (HbSS) patients in steady state. Steady state was defined as subjects who had been free from blood transfusion, crisis and fever for at least 3months, one month and two weeks respectively.[4] Apparently normal individuals with haemoglobin phenotypes HbAS (28 subjects) and HbAA (32 subjects) made up the rest of the recruited subjects and served as controls. Phenotypic, anthropometric and demographic data such as age, sex, weight, height, occupation, average number of pain crisis per year and complications were obtained by questionnaire. Complications were such events like priapism, stroke, gall stones, ankle ulcers and nephropathy.

BMI was determined by anthropometric method as described by Odutola *et al.*[10] Height (m) was measured using a Stadiometer while body weight (kg) was taken using a body weight weighing scale with the subject wearing light clothing and without shoes. Body mass Index (BMI) was calculated as the ratio of weight (kg) to the square of height (m2).

Ethical committee approval and patient's written consent were obtained from the ethics committee of Nnamdi Azikiwe University Teaching Hospital (NAUTH) and the patient respectively.

Disease severity in Hb SS patients

Disease severity was determined by calculating an objective score based on scoring five parameters, pain crisis, anaemia, white cell count, SCD complications and blood transfusion. Scores of ≤3, 3-7 and ≥7 were considered mild, moderate and severe disease respectively, as described by Okocha et al. Pain crisis was defined as pain that necessitated the administration of an oral or parenteral analgesic with or without visit to a chemist shop or medical facility.

Sample collection and laboratory analysis

A total of 5mls of blood was asceptically collected from each subject. 2mls was dispensed into tubes containing Ethylene Diamine Tetraacetic Acid (EDTA) and used to determine Full Blood Count (FBC) and haemoglobin Phenotype. The rest of the sample was dispensed into plain tubes, allowed to clot, centrifuged for 5minutes at 5000rpm. Serum was collected and used to determine GDF-15 levels.

Haemoglobin phenotype was determined by Khon's method (1957) as described by Manafa et al (2017).[11] GDF-15 was determined by sandwich Enzyme Linked Immunosobent assay (ELISA) using commercially available kits (Melsin Medical Co., Limited, Xiaonan Street, Kuancheng District, Changchun 130000, Jilin Province, China) while FBC was determined by the Sysmex method as described by Buttarello and Plebani (2008). Parameters determined included packed cell volume, heamoglobin concentration, white blood cell count and differentials.

Statistical Analysis

Data collected was analysed using Statistical Package for Social Sciences software version 20 (SPSS Inc., IL, Chicago, USA). Values obtained were subjected to statistical analysis using Analysis of variance (ANOVA), Kruskal-Wallis and Student T-test. Person's correlation test was used to determine correlation between variables. P> .05 was considered as significant.

Result

A total of 90 subjects, 48 males and 42 females, with haemoglobin (Hb) phenotypes SS (test), AS and AA (controls); numbering 30, 28 and 32 respectively were studied. Of the 30 Hb SS individuals studied, disease severity was mild in 8 and moderate in 22. The mean ages in years of the different Hb phenotypes SS, AS and AA were 21.57, 22.53 and 23.77 respectively. These were not statistically different, p= .44. However, weight, height and body max index (BMI) were statistically different across the different Hb phenotypes (table 1).

Serum levels of GDF-15 was also significantly different across Hb phenotypes, although the difference between Hb phenotype AS and AA was not significant statistically, p=.05 and .47 respectively. Disease severity negatively correlated with GDF-15 with p value (.098) almost statistically significant (table 3).

Figure 1 is a bar chart comparing the median serum levels of GDF-15 between Hb SS individuals who had mild and moderate disease severity. The difference was statistically significant at p= .01

Table 1: Anthropometric data of different haemoglobin phenotype groups

Groups	Age (years)	Weight (kg)	Height (m)	BMI (kg/m^2)
SS phenotype (A)	21.57±10.90	44.53±17.30	1.54±0.21	17.99±4.09
AS phenotype (B)	22.53±1.98	65.80±9.58	1.70±0.09	22.81±2.26
AA phenotype (C)	23.77±2.70	68.33±8.94	1.74±0.09	22.53±2.15
f-value	0.841	32.624	15.495	24.883
p-value	.435	.001	.001	.001
A vs B	.571	.001	.001	.001
A vs C	.199	.001	.001	.001
B vs C	.470	.436	.245	.718

Table 2: Levels of GDF-15 in different haemoglobin phenotype groups

Groups	GDF-15 (pg/ml)
SS phenotype (A)	60.40
AS phenotype (B)	50.40
AA phenotype (C)	45.15
p-value	0.005

A vs B (p-value)	.038
A vs C (p-value)	.041
B vs C (p-value)	.469

Table 3. Correlation studies of variables with disease severity in subjects with homozygous sickle cell disease (HbSS)

Parameters	N	R	p-value
Disease severity vs age	30	-0.164	.385
Disease severity vs BMI	30	-0.071	.710
Disease severity vs GDF-15	30	-0.307	.098
GDF-15 vs Age	30	-0.099	.602

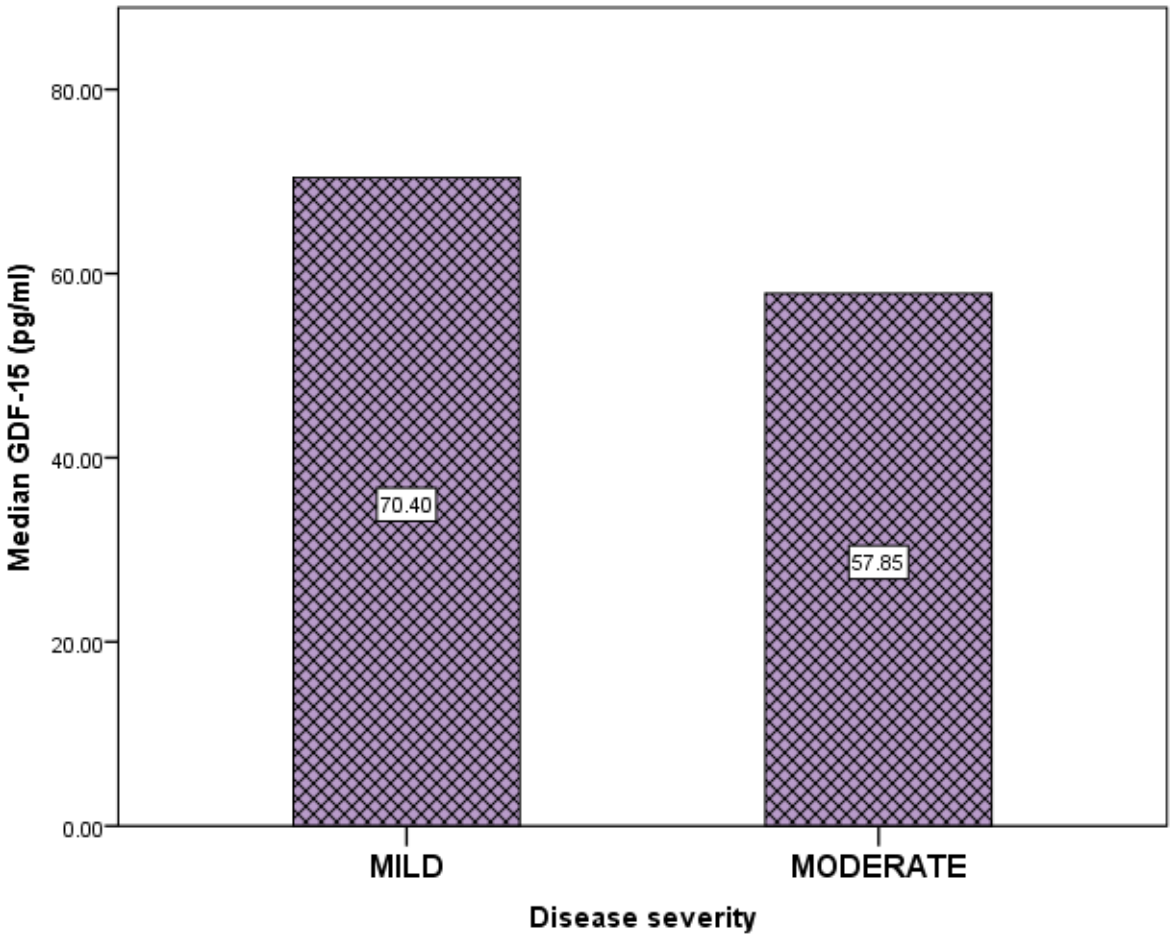

Figure 1: Serum levels of GDF-15 in moderate and mild disease severity in patients with homozygous sickle cell subjects (HbSS) in steady state. p=.010

Discussion

Sickle cell disease (SCD) is an inflammatory disease manifested by elevated leukocyte counts and increased levels of pro-inflammatory cytokines. Inflammatory response has been implicated as a key contributing factor to tissue injury in SCD. Growth differentiation Factor 15 (GDF-15) is an anti-inflammatory cytokine expressed in response to oxidative stress, inflammation and tissue injury. The authors evaluated the levels of GDF-15 in patients with homozygous sickle cell anaemia in steady state and found a significant difference in the median serum level of GDF-15 in the different haemoglobin (Hb) phenotype groups ($P<0.05$). There was a significant increase in the median serum level of GDF-15 in patients with homozygous sickle cell disease (HbSS) compared with the control groups (HbAS & HbAA) ($P<0.05$).

The increase in the levels of GDF-15 in patients with homozygous sickle cell disease in the steady state may be attributed to Ischaemia reperfusion (I/R) injury due to vasooclusive events in homozygous sickle cell disease that triggers an inflammatory response leading to leukocyte mediated microvascular injury not only in the tissue exposed to the initial ischaemic insult, but in other tissues in remote organs. This phenomenon is called the multiorgan dysfunction syndrome (MODS) and is probable part of the reason why there is multiorgan failure in SCD.[12,13] Patients with homozygous sickle cell disease (HbSS) usually exhibit clinical symptoms and complications due to vasooclusion[2] and this may represent a potential stimulus for the release of GDF-15 in order to prevent the progression of inflammation; thus resolving the inflammatory response, promoting the return to homeostasis and inhibiting further tissue damage. The resolution process of the inflammation includes the limitation of neutrophil tissue infiltration, the

counter-regulation of cytokines and chemokines, the induction of apoptosis in spent neutrophils and their efferocytosis by macrophages.[14]

In our data set we found the range of serum GDF15 in apparently normal individuals to be 16 - 691 pg/ml; the level rises dramatically when disease develops. This quick up regulation of GDF-15 is aimed at preventing cells from further damage.[15] GDF-15 probably does this by inhibiting the inflammatory response that predominantly involves neutrophil infiltration and trans-endothelial migration via blocking the activation of β2 integrin affinity and clustering thereby preventing neutrophil adhesion to ICAM-1.[16] This is a protective effect against warm I/R injury.[17]

GDF-15 seems to be an adaptational response to periods of ischaemia in tissues which if blunted in the steady state, may lead to increased severity in disease; hence in our data set, SCD patients who had a reduced median serum level of GDF-15 had disease of moderate severity compared to those who had higher median serum levels and presented with mild disease $p = 0.01$ (fig.1). This blunting may be because of increased ischaemic damage in SCD which may impair organ functions, leading to decreased expression of anti-inflammatory cytokines. We hypothesize that GDF -15 may be a potential therapeutic target for intervention against ischaemia/reperfusion induced microvascular injury; since it modulates generation of reactive oxygen specie (ROS) thus maintaining cellular homeostasis.

Polyphenols in fruits and vegetables that function similarly are gaining growing interest as therapy in ROS related diseases. Tsumbu et al, evaluated Cassava [Manihot esculenta Crantz (MEC)] leaves - consumed by more than 200 million inhabitants in sub-Saharan Africa as food and folk medicine- in this regard and concluded that the role of this plant in the prevention of

oxidative stress in ROS related diseases is worthy of further consideration. They found that the aqueous extract of MEC leaves had significant antiradical and antioxidant activities which were not impaired by low heat treatment.[18,19] Baheka and Khale also found that MEC leave extracts significantly increased the serum levels of antioxidant enzymes such as superoxide dismutase, reduced glutathione and catalase.[20] Some of these enzymes have been found to be low in SCD.[5]

The finding of this present study supports the observation of Papassotiriou et al.[21] who found that GDF-15 levels were elevated in patients with HbS/β-thal compared with the control group ($p<0.001$) and that it was negatively correlated with Hepcidin-25/Ferritin molar ratio. They also found GDF-15 elevated in SCD patients who had been on hydroxyurea therapy. Tantawy *et al.* (2014) also concluded that GDf-15 levels were increased in sickle cell disease patients whether sickle cell anaemia or sickle-β thalassaemia compared with controls ($p<0.001$). They however did not find GDF-15 significantly related to some indices of SCD severity such as frequency of sickling crisis and pulmonary hypertension.[22] We note here that the tool with which we scored for SCD severity was objective and robust, since it was a composite of multiple indices. This may have accounted for the difference between our work and that of Tantawy et al where the authors correlated GDF-15 levels with single indices of SCD severity. This work is limited by the fact that it was done in a single centre. The results should therefore be confirmed by a multicentre study with a higher number of subjects.

In conclusion, the authors have found serum levels of GDF-15, which is an anti inflammatory cytokine, to be increased in SCD compared to controls and to correlate negatively with disease severity. It may be a potential therapeutic target for intervention against ischaemia/reperfusion induced microvascular injury.

REFERENCE

1. Human Genomics in Global

Health. https://www.who.int/genomics/public/geneticdiseases/en/index2.html, 2019.

(Assessed 27th April 2019)

2. Ademola Samson Adewoyin, "Management of Sickle Cell Disease: A Review for Physician Education in Nigeria (Sub-Saharan Africa)," Anemia, vol. 2015, Article ID 791498, 21 pages. https://doi.org/10.1155/2015/791498.

3. Elguero E, Délicat-Loembet LM, Rougeron V, Arnathau C, Roche B, Becquart P, et al. Malaria continues to select for sickle cell trait in Central Africa. *Proc Natl Acad Sci U S A*. 2015 Jun 2; 112(22):7051-4. doi: 10.1073/pnas.1505665112. Epub 2015 May 4. PubMed PMID: 25941403; PubMed Central PMCID: PMC4460506.

4. CE Okocha, PO Manafa, JO Ozomba, TO Ulasi, GO Chukwuma, JC Aneke. C-reactive protein and disease outcome in Nigerian sickle cell disease patients. Ann Med Health Sci Res.2014;4:701-5. doi: 10.4103/2141-9248.141523. PMID: 25328778, PMCID PMC4199159

5. EC Okocha, OP Manafa, CJ Aneke, EC Onwuzuruike, CN Ibeh, ...Serum superoxide dismutase activity: A predictor of disease severity in Nigerian sickle cell anemia patients in steady state. Med J DY Patil Univ. 2017; 10 (5): 406-411. **DOI:** 10.4103/MJDRDYPU.MJDRDYPU_90_17

6. Manafa P, Okocha C, Nwogho B, et al. comparative study of carbohydrate antigen 19-9 in sickle cell disease subjects and controls in Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria. *Afr Health Sci*. 2018;18(4):1003–1009. doi:10.4314/ahs.v18i4.21

7. Ago T and Sadoshima J. GDF15, a cardioprotective TGF-β superfamily protein. Circ Res. 2006; 98(3):294-7. PMID:16484622 DOI: 10.1161/01.RES.0000207919.83894.9d

8. Kempf T, Eden M, Strelau J, Naguib M, Willenbockel C, Tongers J, et al. Transforming growth factor-{beta} superfamily member growth-differentiation factor-15 protects the heart from ischemia/reperfusion injury. *Circ Res.* 2006;98 (3) :351–360. PMID: 16397141 DOI: 10.1161/01.RES.0000202805.73038.48

9. Robert P. Hebbel, Ischemia-reperfusion Injury in Sickle Cell Anemia: Relationship to Acute Chest Syndrome, Endothelial Dysfunction, Arterial Vasculopathy, and Inflammatory Pain. Hematol. Oncol. Clin. North. Am. 2014;28(2):181-98. PMID: 24589261 DOI: 10.1016/j.hoc.2013.11.005

10. Odetunde, I.O, Chinawa JM., Achigbu KI., Achigbu, EO. Body mass index and other anthropometric variables in children with sickle cell anaemia. Pak. J. Med. Sci.; 2016:32(2): 341–46. DOI: 10.12669/pjms.322.9046 PMID: 27182236 PMCID: PMC4859019

11. PO Manafa, CE Okocha, JC Aneke, U Obiano, NC Ibeh, GO Chukwuma. Low serum glutathione-S-transferase activity and vitamin E levels do not correlate with disease severity in steady state adults with sickle cell anemia. J. Appl. Hematol. 2017:8 (3): 110-15. **DOI:** 10.4103/joah.joah_22_17

12. Carden, D. L. and Granger, D. N. Pathophysiology of ischaemia–reperfusion injury. J. Pathol., 2000;190 (3): 255-66. doi:10.1002/(SICI)1096-9896(200002)190:3<255::AID-PATH526>3.0.CO;2-6

13. Kato, G. J., Hebbel, R. P., Steinberg, M. H., Gladwin, M. T. Vasculopathy in sickle cell disease: Biology, pathophysiology, genetics, translational medicine, and new research directions. Am J Haematol. 2009;84 (9): 618-25. PMID:19610078 PMCID: PMC3209715 DOI: 10.1002/ajh.21475

14. Headland SE, Norling LV. The resolution of inflammation: Principles and Challenges. Semin Immunol.2015;27(3):149–60. PMID: 25911383 DOI: 10.1016/j.smim.2015.03.014

15. Kempf, T., Eden, M., Strelau, J., Naguib, M., Willenbockel, C., Tongers, J, et al. The transforming growth factor-beta superfamily member growth-differentiation factor-15 protects the heart from ischemia/reperfusion injury. Circ Res 2006; 98:351–60. PMID: 16397141 DOI: 10.1161/01.RES.0000202805.73038.48

16. Kempf T, Zarbock A, Widera C, Butz S, Stadtmann A, Rossaint J, *et al*: GDF-15 is an inhibitor of leukocyte integrin activation required for survival after myocardial infarction in mice. Nat Med 2011;17 (5): 581-8. PMID: 21516086 DOI: 10.1038/nm.2354

17. Zhang, M., Pan, K., Liu, Q., Zhou, X., Jiang, T., Li, Y. Growth differentiation factor 15 may protect the myocardium from no-reflow by inhibiting the inflammatory-like response that predominantly involves neutrophil infiltration. *Mol Med Rep* 2016; 13 (1) :623–32. https://doi.org/10.3892/mmr.2015.4573

18. Tsumbu, C.N.; Deby-Dupont, G.; Tits, M.; Angenot, L.; Franck, T.; Serteyn, D. et al. Antioxidant and antiradical activities of *Manihot esculenta* Crantz

(Euphorbiaceae) leaves and other selected tropical green vegetables investigated on lipoperoxidation and PMA activated monocytes. *Nutrients* **2011**; *3(9):* 818–38. PMID: 22254126 PMCID: PMC3257738 DOI: 10.3390/nu3090818

19. Tsumbu, C.N., Deby-Dupont, G., Tits, M., Angenot, L., Frederich, M., Kohnen, S., et al. Polyphenol content and modulatory activities of some tropical dietary plant extracts on the oxidant activities of neutrophils and myeloperoxidase. Int J Mol Sci 2012; 13: 628–50. doi: 10.3390/ijms13010628 PMCID: PMC3269710 PMID: 22312276

20. Bahekar SE, Kale RS. Evaluation of antioxidant activity of *Manihot esculenta* Crantz in wistar rats. J Pharm Bioallied Sci 2016;8 (2):119-23 PMID: 27134463 PMCID: PMC4832901 DOI: 10.4103/0975-7406.171697

21. Larissi K, Politou M, Margeli A, Poziopoulos C, Flevari P, Terpos E. .The Growth Differentiation Factor-15 Levels Are Increased in Patients with Compound Heterozygous Sickle Cell and Beta-Thalassemia, Correlate with Hepcidin-25/Ferritin Molar Ratio and with Markers of Hemolysis, Endothelial Dysfunction and Angiogenesis. *Blood Cell Mol Dis*.2019; 77:137-41. doi: 10.1016/j.bcmd.2019.04.011. PMID: 31071550

22. Tantawy AA, Adly AA, Ismail EA, Darwish YW, Ali Zedan M Growth differentiation factor-15 in young sickle cell disease patients: relation to hemolysis, iron overload and vascular complications. Blood Cells Mol Dis 2014; 53(4):189–93. PMID: 25065856 DOI: 10.1016/j.bcmd.2014.07.003