April 2022 ISSN 1650-3414 Volume 33 Number 1

eJIFCC

Communications and Publications Division (CPD) of the IFCC

Editor-in-chief: Prof. János Kappelmayer, MD, PhD
Faculty of Medicine, University of Debrecen, Hungary
e-mail: ejifcc@ifcc.org

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

In this issue

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

This is a Platinum Open Access Journal distributed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Klotho: a possible role in the pathophysiology of nephrotic syndrome

Sojit Tomo[1], Amandeep Birdi[1] , Dharmveer Yadav[1], Manish Chaturvedi[2], Praveen Sharma[1]

[1] *Department of Biochemistry, All India Institute of Medical Sciences, Jodhpur, Rajasthan, India*

[2] *Department of Nephrology, All India Institute of Medical Sciences, Jodhpur, Rajasthan, India*

ARTICLE INFO

Corresponding author:
Dr. Dharmveer Yadav
Associate Professor
Department of Biochemistry
All India Institute of Medical Sciences, Jodhpur, Rajasthan
India
E-mail: dharam143s@gmail.com

Key words:
Klotho, nephrotic syndrome, proteinuria, angiotensinogen, oxidative stress

ABSTRACT

Klotho, encoded by the *klotho* gene, is associated with phosphate homeostasis. Klotho acts as a co-receptor for FGF23 for binding to its receptors. With FGF23, klotho regulates the systemic mineral homeostasis by regulation of vitamin D and parathyroid hormone. The anti-inflammatory, antifibrotic and antioxidant properties of klotho give it a cardinal role in the development of various renal diseases. The protective effect of klotho has been evident in different types of nephropathy, including diabetic nephropathy, cyclosporine A-induced nephropathy, Calcineurin inhibitors-induced nephropathy, and renal ischemic-reperfusion injury. Nephrotic syndrome is distinguished by hypoproteinemia, proteinuria, and hypercholesterolemia as a result of the aberration of the glomerular filtration barrier. The various factors and pathways associated with the pathophysiology of the nephrotic syndrome have similarities with other types of nephropathy. Despite these similarities,

the role of klotho in the pathology of nephrotic syndrome remains still unexplored. This mini-review builds the case for the possible role of klotho in nephrotic syndrome. The review explores the possible pathways where klotho can play a major role by identifying the similarities in the pathophysiology of nephrotic syndrome and other types of nephropathy.

❖❖❖❖❖

INTRODUCTION

Klotho, a 135 kDa transmembrane protein, is associated with the aging process and is involved in phosphate metabolism, and regulates the activity of fibroblast growth factors (FGF) [1]. The word klotho has been derived from the name of the Greek goddess Clotho who is believed to spin the "thread" of human fate. The derivation of the word signifies a metaphor for the life span of an individual as the *klotho* gene (KL) is associated with aging. The role of klotho in aging was first reported in mouse studies where KL deficient mice had a decreased life span of fewer than eight weeks [2]. Similarly, an increased expression of the KL gene had positive effects on the life span of the organism [3]. Animal studies demonstrated an association between klotho protein deficiency and an increase in phosphate levels in the blood that is attributed to be one of the mechanisms involved in the decreasing life span in the animals.

The changes observed in klotho deficient animals are comparable to the age-related senile changes observed in humans [4, 5]. These major changes included plaque formation in the arteries, weakening of bones, fat and muscle loss, hasty shrinkage of the thymus resulting in changes in the architecture of the thymus, and decrease in tissue mass leading to gradual deterioration of the immune system; which are similar to senile changes observed in the elderly.

The klotho gene family comprises α-klotho, β-klotho, and γ-klotho [6]. α-KL gene, located on Chromosome 13, comprises three exons and two introns [7] and encodes a transmembrane protein with a smaller cytoplasmic domain and a larger extracellular domain. There are three distinct types of α-klotho protein: transmembrane klotho, secretory klotho, and soluble klotho [7]. Metalloproteinases enzymes ADAM 10 and 17 act on the extracellular domain of klotho to release it from its membrane sites [8-10]. The enzymatic cleavage by the sheddase enzyme at the extracellular domain leads to the generation of soluble klotho form. Secretory klotho, having a molecular weight of 70 kDa, is another form of klotho protein formed by alternate splicing of KL exons. Fibroblast Growth Factor-23 (FGF23) plays a crucial role in phosphate and vitamin D metabolism requires transmembrane α-klotho protein as its co-receptor [11]. β-klotho and γ-Klotho are also transmembrane proteins [6]. β-klotho acts as a co-receptor for FGF19 and FGF21 regulating bile acid synthesis and energy metabolism [12]. Both the transmembrane form of α-klotho protein (acting as a co-receptor for FGF23) and the soluble form of klotho has been demonstrated to be involved in pathways whose aberration can lead to nephrogenic effects [13,14].

Klotho has been directly implicated in the development of chronic kidney disease (CKD) [15]. A decrease in klotho levels succeeded by a rise in serum FGF23 indicated deterioration of kidney function in chronic kidney disease. Further, FGF23, produced from bones, regulates mineral metabolism [11].

Increased excretion of protein in urine and the resultant edema and hypoalbuminemia characterize the nephrotic syndrome. Of all types of nephrotic syndrome, some are steroid-resistant and some are steroid responsive. Minimal change disease, the commonest cause, is steroid responsive.

Steroid resistance includes focal segmental glomerulosclerosis that has a significant risk of kidney failure [16]. Systemic diseases such as Lupus can also cause nephrotic syndrome.

The primary and secondary causes of nephrotic syndrome should be distinguished and management strategies appropriately tailored. Immunosuppressive medications are the mainstay of treatment. The various causes of nephrotic syndrome have been categorized and listed in Table 1.

Klotho has significant beneficial effects on the kidney by alleviating oxidative stress and by its antiapoptotic properties. This protective effect of klotho in different types of nephropathy, including diabetic nephropathy, has been demonstrated in multiple studies.

The pathologic alterations observed in different types of nephropathy such as increased oxidative stress, and inflammation, have also been demonstrated in nephrotic syndrome. However few studies have explored the role of klotho in nephrotic syndrome.

KLOTHO IN DIABETIC NEPHROPATHY

The role of klotho in the development of diabetic nephropathy has been explored in various animal models. A decreased klotho level in animal models increased the purinergic receptor $P2X_7$ culminating in cell death by apoptosis or necrosis in diabetic nephropathy [18]. Klotho was also demonstrated to suppress the hyperglycemia-mediated glomerular endothelial injury and activation of the Wnt/β-catenin pathway in mice models of diabetic nephropathy [19]. The aetiological role of klotho in DN was further expounded by the attenuation of apoptosis of renal tubular cells by the drug atrasentan that acts by decreasing the expression of miR-199b-5p and thus increasing its target, klotho [20]. A reduction in the odds of early nephropathy in T2DM patients was observed at a higher concentration of FGF-23, which correlated positively with sKL in diabetic patients [21]. Interestingly, nephrotic syndrome, when compared with controls is associated with decreased levels of FGF23. The reduction in Vitamin D levels and the loss of FGF23 in urine are thought to contribute to lowered levels observed [22].

Table 1 List of various causes of nephrotic syndrome categorized based on etiology

Genetic	Infectious causes	Idiopathic	Others
• Diffuse mesangial sclerosis (DMS) • Epidermolysis bullosa associated • Steroid-resistant nephrotic syndrome • Familial focal segmental glomerulosclerosis (FSGS)	• Congenital infections including syphilis, toxoplasmosis, and HIV • Cytomegalovirus • HIV-associated nephropathy	• Minimal change nephropathy • Focal segmental glomerulosclerosis • Diffuse mesangial hypercellularity • Membranous glomerulonephritis • Membranoproliferative GN	• Lupus nephropathy • IgA nephropathy • Drugs • Malignancies • Hemolytic uremic syndrome (HUS)

INFLAMMATION AND OXIDATIVE STRESS IN NEPHROPATHY

The protective effect of klotho has also been explored in other types of nephropathy. Increased klotho levels, in cyclosporine A-induced nephropathy, regulate cytokine expression and modulate the inflammation via PDLIM2/NF-kB p65 pathway resulting in beneficial effects [23]. It also has a crucial role in protection against Calcineurin inhibitors-induced nephropathy [24]. The decreased klotho levels make the kidney vulnerable to oxidative stress-induced organ injury [25]. Klotho mitigates oxidative stress by increasing the manganese superoxide dismutase expression via suppression of the PI3K-AKT signaling pathway [26]. Further, klotho also prevents Calcineurin inhibitors-induced nephropathy by improving autophagy clearance and preventing autophagy cell death [27]. In renal ischemic-reperfusion injury, klotho was demonstrated to have an inhibitory effect on oxidative stress in tubular epithelial cells thus preventing necroptosis [28]. Besides, the inflammatory environment also adversely affects the klotho expressions via NFκB-dependent mechanism as shown by the downregulation of klotho expression by inflammatory cytokines, such as TWEAK and TNFα [29].

Similar to the aforementioned types of nephropathy, the nephrotic syndrome also has been correlated with increased oxidative stress and inflammatory state. Patients with steroid-sensitive nephrotic syndrome had higher plasma levels of advanced oxidation protein products and malondialdehyde indicating oxidative stress in them [30]. The increased pro-oxidant status in nephrotic children leads to considerable change in antioxidant concentrations [31]. The presence of oxidative stress and abnormality in the antioxidative system has been verified in adult nephrotic syndrome patients also [32, 33]. The increased activity of GSH-Px and selenium content in polymorphonuclear leukocytes (PMNLs) in nephrotic syndrome also indicates the presence of oxidative stress in these patients [34]. The decrease in NF-kappaB p65 in addition to the up-regulation of IL-2 are mechanisms hypothesized to initiate glucocorticoid resistance in steroid-resistant nephrotic syndrome [35]. Similarly, elevated serum TNFα levels in nephrotic syndrome are associated with a lack of response to steroids [36]. Further, In vitro study has demonstrated the increased expression by TWEAK of PLA2R as well as NFKB1 and IRF4 which are linked to membranous nephropathy [37].

FACTORS AFFECTING KLOTHO EXPRESSION

Albuminuria, in cultured tubular cells, decreased the expression of klotho [38]. Concurrently, in CKD animal models with frank albuminuria, the klotho expressions were found to be suppressed indicating a possible role that klotho may have in the pathogenesis of CKD.

Proteinuric kidney disease is associated with endoplasmic reticulum (ER) stress. Animal models of albuminuria demonstrated features of ER stress in renal tubular cells which are instigated by the albumin and mediated via ATF3/ATF4 activation. The induction of ATF3 and ATF4 leads to enhanced binding to the promoter region effectuating altered transcription of the klotho gene culminating in the suppression of klotho expression [39]. ATF3 is also induced in renal ischemia-reperfusion injury, where klotho is observed to be downregulated [40]. Further, ER stress accentuates klotho degradations via proteasome and lysosome. Hence, proteinuria (especially albuminuria) leads to decreased klotho protein half-life as well as the genetic expression [41].

Further, the downregulation of klotho in tubular epithelial cells is also associated with the aggravation of renal fibrosis [42]. Although solu-

ble α-klotho positively correlated with eGFR in patients with CKD, the efficacy of klotho in improving renal function in CKD patients is still under investigation [43]. Further, the expression of klotho in kidneys is also affected by excessive renin–angiotensin–aldosterone system (RAAS) activation. In vitro experiments demonstrated Angiotensin II to have a suppressive effect on klotho through PPAR-γ downregulation [44] (Figure 1).

The aforementioned factors affecting klotho expression (albuminuria and Angiotensin II) have been implicated in the pathophysiology of nephrotic syndrome in various studies. The glomerular-derived angiotensinogen has been thought to have a significant effect on glomerular dysfunction in nephrotic syndrome. As a result, the ARB treatment is beneficial in slit diaphragm injury by inhibiting the positive feedback loop of the activated local Ang II action [45]. During podocyte injury, a vicious loop that stimulates the intrarenal generation of Angiotensin II is activated aggravating the development of glomerular dysfunction [46]. Further, PPARγ agonists decrease the proteinuria in acute nephrotic syndrome by regulating the expression of multiple genes like actinin-4 and nephrin and leading to the restoration of podocyte structure [47]. Hence, activation of PPARγ and inhibition of ANGPTL4 is associated with a better prognosis in patients with nephrotic syndrome [48].

CONCLUSION

Figure 2 summarizes the possible mechanisms by which klotho may play a cardinal role in the pathophysiology of nephrotic syndrome.

The various factors associated with the pathophysiology of nephrotic syndrome, including oxidative stress and inflammation, are intertwined with klotho expression and its downstream effects. The multitude of studies demonstrating the association of klotho and these factors in other types of nephropathy warrants its investigation into the nephrotic syndrome. The insights thus obtained would help in designing therapeutic strategies involving klotho and its downstream effectors for nephrotic syndrome.

Figure 1 **Depiction of factors and their modes of influencing the expression of klotho. A: Albuminuria; B: Angiotensin II**

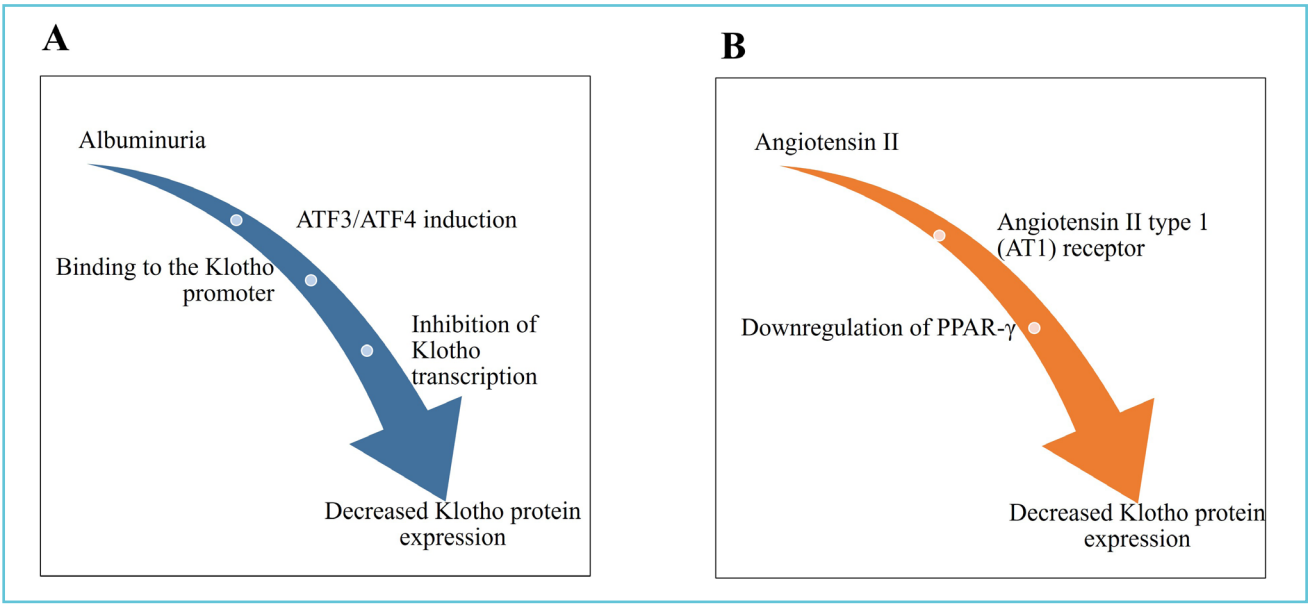
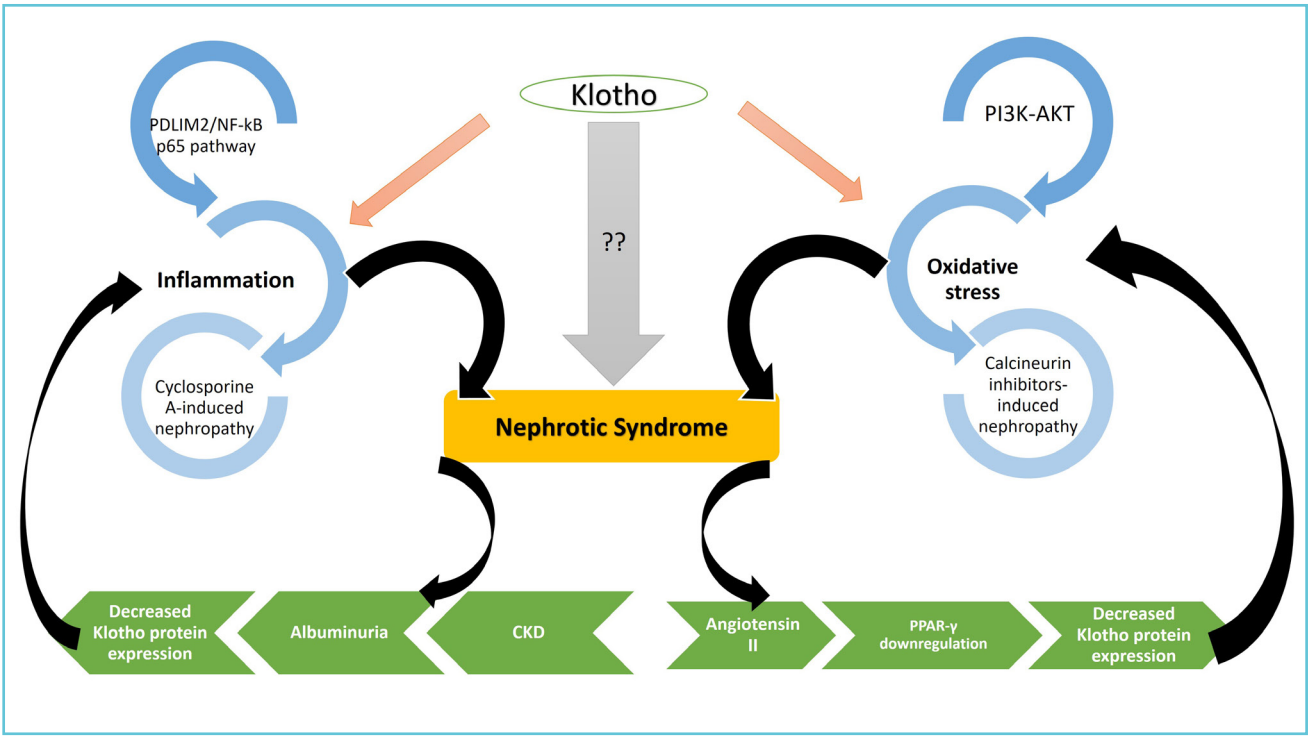


Figure 2 Illustration of the possible roles of klotho in nephrotic syndrome



REFERENCES

1. Vo HT, Laszczyk AM, King GD. Klotho, the Key to Healthy Brain Aging?. Brain Plast 2018;3(2):183-194.

2. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse KLOTHO gene leads to a syndrome resembling ageing. Nature. 1997; 390(6655):45–51.

3. Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, et al. Suppression of aging in mice by the hormone KLOTHO. Science.2005;309(5742):1829–33.

4. Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, et al. KLOTHO converts canonical FGF receptor into a specific receptor for FGF23. Nature. 2006;444(7120): 770–74.

5. Kurosu H, Ogawa Y, Miyoshi M, Yamamoto M, Nandi A, Rosenblatt KP, et al. Regulation of fibroblast growth factor-23 signaling by KLOTHO. J Biol Chem. 2006;281(10): 6120–3.

6. Ito S, Fujimori T, Hayashizaki Y, Nabeshima Y. Identification of a novel mouse membrane-bound family 1 glycosidase-like protein, which carries an atypical active site structure. Biochim Biophys Acta. 2002;1576:341–5.

7. Shiraki-Iida T, Aizawa H, Matsumura Y, Sekine S, Iida A, Anazawa H, et al. Structure of the mouse KLOTHO gene and its two transcripts encoding membrane and secreted protein. FEBS Lett. 1998;424(1-2):6-10.

8. Imura A, Tsuji Y, Murata M, Maeda R, Kubota K, Iwano A, et al. alpha-KLOTHO as a regulator of calcium homeostasis. Science. 2007;316(5831):1615–8.

9. Chen C-D, Podvin S, Gillespie E, Leeman SE, Abraham CR. Insulin stimulates the cleavage and release of the extracellular domain of KLOTHO by ADAM10 and ADAM17. Proc Natl Acad Sci U S A. 2007;104(50):19796-801.

10. Chen C-D, Tung TY, Liang J, Zeldich E, Tucker Zhou TB, Turk BE, et al. Identification of cleavage sites leading to the shed form of the anti-aging protein KLOTHO. Biochemistry. 2014;53(34):5579-87.

11. Navarro-García JA, Fernández-Velasco M, Delgado C, Delgado JF, Kuro-O M, Ruilope LM, et al. PTH, vitamin D, and the FGF-23-klotho axis and heart: Going beyond the confines of nephrology. Eur J Clin Invest 2018; 48(4).

12. Kurosu H, Kuro OM. The klotho gene family as a regulator of endocrine fibroblast growth factors. Mol Cell Endocrinol. 2009; 299:72–8

13. Zou, D., Wu, W., He, Y. et al. The role of klotho in chronic kidney disease. BMC Nephrol. 2018;19:285.

14. Dalton GD, Xie J, An SW, Huang CL. New Insights into the Mechanism of Action of Soluble Klotho. Front Endocrinol (Lausanne). 2017;8:323

15. Komaba H, Lanske B. Role of Klotho in bone and implication for CKD. Curr Opin Nephrol Hypertens 2018;27(4): 298-304.

16. Wang CS, Greenbaum LA. Nephrotic Syndrome. Pediatr Clin North Am 2019;66(1):73-85.

17. Ranganathan S. Pathology of Podocytopathies Causing Nephrotic Syndrome in Children. Front Pediatr. 2016 Mar 31;4:32.

18. Rodrigues AM, Serralha RS, Farias C, Punaro GR, Fernandes MJS, Higa EMS. P2X7 receptor and klotho expressions in diabetic nephropathy progression. Purinergic Signal 2018;14(2):167-176.

19. Wang Q, Ren D, Li Y, Xu G. Klotho attenuates diabetic nephropathy in db/db mice and ameliorates high glucose-induced injury of human renal glomerular endothelial cells. Cell Cycle 2019;18(6-7):696-707.

20. Kang WL, Xu GS. Atrasentan increased the expression of klotho by mediating miR-199b-5p and prevented renal tubular injury in diabetic nephropathy. Sci Rep 2016;6: 19979.

21. Farías-Basulto A, Martínez-Ramírez HR, Gómez-García EF, Cueto-Manzano AM, Cortés-Sanabria L, Hernández-Ramos LE, et al. Circulating Levels of Soluble Klotho and Fibroblast Growth Factor 23 in Diabetic Patients and Its Association with Early Nephropathy. Arch Med Res 2018;49(7):451-455.

22. Yadav AK, Ramachandran R, Aggarwal A, Kumar V, Gupta KL, Jha V. Fibroblast growth factor 23 in untreated nephrotic syndrome. Nephrology (Carlton) 2018;23(4): 362-365.

23. Jin M, Lv P, Chen G, Wang P, Zuo Z, Ren L, et al. Klotho ameliorates cyclosporine A-induced nephropathy via PDLIM2/NF-kB p65 signaling pathway. Biochem Biophys Res Commun 2017;486(2):451-457.

24. Jin J, Jin L, Lim SW, Yang CW. Klotho deficiency aggravates tacrolimus-induced renal injury via the phosphatidylinositol 3-kinase-Akt-forkhead box protein O pathway. Am J Nephrol 2016;43(5):357–365.

25. Luo K, Lim SW, Quan Y, Cui S, Shin YJ, Ko EJ, et al. Role of Klotho in Chronic Calcineurin Inhibitor Nephropathy. Oxid Med Cell Longev. 2019;2019:1825018.

26. Lim SW, Jin L, Luo K, Jin J, Shin YJ, Hong SY, et al. Klotho enhances FoxO3-mediated manganese superoxide dismutase expression by negatively regulating PI3K/AKT pathway during tacrolimus induced oxidative stress. Cell Death Dis 2017;8(8):e2972.

27. Lim SW, Shin YJ, Luo K, Quan Y, Ko EJ, Chung BH, Yang CW. Effect of Klotho on autophagy clearance in tacrolimus-induced renal injury. FASEB J 2019;33(2):2694-2706.

28. Qian Y, Guo X, Che L, Guan X, Wu B, Lu R, et al. Klotho Reduces Necroptosis by Targeting Oxidative Stress Involved in Renal Ischemic-Reperfusion Injury. Cell Physiol Biochem 2018;45(6):2268-2282.

29. Moreno JA, Izquierdo MC, Sanchez-Niño MD, Suárez-Alvarez B, Lopez-Larrea C, Jakubowski A, et al. The inflammatory cytokines TWEAK and TNFα reduce renal klotho expression through NFκB. J Am Soc Nephrol 2011;22(7): 1315-25.

30. Fan A, Jiang X, Mo Y, Tan H, Jiang M, Li J. Plasma levels of oxidative stress in children with steroid-sensitive nephrotic syndrome and their predictive value for relapse frequency. Pediatr Nephrol 2016;31(1):83-8.

31. Kamireddy R, Kavuri S, Devi S, Vemula H, Chandana D, Harinarayanan S, et al. Oxidative stress in pediatric nephrotic syndrome. Clin Chim Acta 2002;325(1-2):147-50.

32. Soyoral YU, Aslan M, Emre H, Begenik H, Erdur FM, Turkel A, et al. Serum paraoxonase activity and oxidative stress in patients with adult nephrotic syndrome. Atherosclerosis 2011;218(1):243-6.

33. Bulucu F, Vural A, Aydin A, Sayal A. Oxidative stress status in adults with nephrotic syndrome. Clin Nephrol 2000; 53(3):169-73.

34. Akyol T, Bulucu F, Sener O, Yamanel L, Aydin A, Inal V, et al. Functions and oxidative stress status of leukocytes in patients with nephrotic syndrome. Biol Trace Elem Res 2007;116(3):237-48.

35. Aviles DH, Matti Vehaskari V, Manning J, Ochoa AC, Zea AH. Decreased expression of T-cell NF-kappaB p65 subunit in steroid-resistant nephrotic syndrome. Kidney Int 2004;66(1):60-7.

36. Weissbach A, Garty BZ, Lagovsky I, Krause I, Davidovits M. Serum Tumor Necrosis Factor-Alpha Levels in Children with Nephrotic Syndrome: A Pilot Study. Isr Med Assoc J 2017;19(1):30-33.

37. Cuarental L, Valiño-Rivas L, Mendonça L, Saleem M, Mezzano S, Sanz AB, et al. Tacrolimus Prevents TWEAK-Induced PLA2R Expression in Cultured Human Podocytes. J Clin Med 2020;9(7):2178.

38. Fernandez-Fernandez B, Izquierdo MC, Valiño-Rivas L, Nastou D, Sanz AB, Ortiz A, Sanchez-Niño MD. Albumin downregulates Klotho in tubular cells. Nephrol Dial Transplant 2018;33(10):1712-1722.

39. de Seigneux S, Wilhelm-Bals A, Courbebaisse M. On the relationship between proteinuria and plasma phosphate. Swiss Med Wkly 2017;147:w14509.

40. Hu MC, Shi M, Zhang J, Quiñones H, Kuro-o M, Moe OW. Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. Kidney Int 2010;78(12):1240-51.

41. Delitsikou V, Jarad G, Rajaram RD, Ino F, Rutkowski JM, Chen CD, et al. Klotho regulation by albuminuria is dependent on ATF3 and endoplasmic reticulum stress. FASEB J 2020;34(2):2087-2104.

42. Liu Y, Bi X, Xiong J, Han W, Xiao T, Xu X, et al. MicroRNA-34a Promotes Renal Fibrosis by Downregulation of Klotho in Tubular Epithelial Cells. Mol Ther 2019;27(5): 1051-1065.

43. Wang Q, Su W, Shen Z, Wang R. Correlation between Soluble α-Klotho and Renal Function in Patients with Chronic Kidney Disease: A Review and Meta-Analysis. Biomed Res Int 2018;2018:9481475.

44. Maquigussa E, Paterno JC, de Oliveira Pokorny GH, da Silva Perez M, Varela VA, da Silva Novaes A, et al. Klotho and PPAR Gamma Activation Mediate the Renoprotective Effect of Losartan in the 5/6 Nephrectomy Model. Front Physiol 2018;9:1033.

45. Yamazaki M, Fukusumi Y, Kayaba M, Kitazawa Y, Takamura S, Narita I, et al. Possible role for glomerular-derived angiotensinogen in nephrotic syndrome. J Renin Angiotensin Aldosterone Syst 2016;17(4):1470320316681223.

46. Koizumi M, Ueda K, Niimura F, Nishiyama A, Yanagita M, Saito A, et al. Podocyte Injury Augments Intrarenal Angiotensin II Generation and Sodium Retention in a Megalin-Dependent Manner. Hypertension 2019;74(3): 509-517.

47. Zuo Y, Yang HC, Potthoff SA, Najafian B, Kon V, Ma LJ, et al. Protective effects of PPARγ agonist in acute nephrotic syndrome. Nephrol Dial Transplant 2012;27(1):174-81.

48. Lu R, Zhou J, Liu B, Liang N, He Y, et al. Paeoniflorin ameliorates Adriamycin-induced nephrotic syndrome through the PPARγ/ANGPTL4 pathway in vivo and vitro. Biomed Pharmacother 2017;96:137-147.

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

This is a Platinum Open Access Journal distributed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Novel damage biomarkers of sepsis-related acute kidney injury

Dániel Ragán[1,2], Zoltán Horváth-Szalai[1,3], Balázs Szirmay[1], Diána Mühl[2]

[1] *Department of Laboratory Medicine, Medical School, University of Pécs, Hungary*
[2] *Department of Anesthesiology and Intensive Therapy, Medical School, University of Pécs, Hungary*
[3] *János Szentágothai Research Center, University of Pécs, Hungary*

ARTICLE INFO

Corresponding author:
Dániel Ragán
Department of Laboratory Medicine
Medical School, University of Pécs
H-7624 Pécs, Ifjúság u. 13
Hungary
Phone: +36 30 364 0402
Fax: +36 72 536 121
E-mail: ragandaniel@hotmail.com

Key words:
Sepsis-3, acute kidney injury, presepsin, gelsolin, urinary actin, urinary Gc-globulin, novel biomarker

ORCID ID:
https://orcid.org/0000-0002-5429-0168

ABSTRACT

Sepsis-related acute kidney injury (AKI) is one of the most common complications of sepsis at the intensive care unit (ICU) with more adverse mortality rates. The early diagnosis and reliable monitoring of sepsis-related AKI are essential in achieving a favorable outcome. Novel serum and urinary biomarkers could yield valuable information during this process.

Regarding the widely used Kidney Disease Improving Global Outcomes (KDIGO) classifications, the diagnosis of AKI is still based on the increase of serum creatinine levels and the decrease of urine output; however, these parameters have limitations in reflecting the extent of kidney damage, therefore more sensitive and specific laboratory biomarkers are needed for the early diagnosis and prognosis of sepsis-related AKI. Regarding this, several serum parameters are discussed in this review including presepsin and the most important actin scavenger proteins (gelsolin,

Gc-globulin) while other urinary markers are also examined including cell cycle arrest biomarkers, neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), Cystatin C and actin.

Novel biomarkers of sepsis-related AKI could facilitate the early diagnosis and monitoring of sepsis-related AKI.

INTRODUCTION

Sepsis is a complex clinical syndrome with increasing incidence and unfavorable mortality rates which still poses a significant challenge in intensive care despite the availability of advanced treatment methods [1-3]. As stated in the latest Sepsis-3 definitions, sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection [4].

CONVENTIONAL SEPSIS BIOMARKERS

Timely diagnosis along with effective causal and supportive treatment of sepsis are essential for achieving adequate recovery. Although not being part of the Sepsis-3 definitions, serum high-sensitivity C-reactive protein (CRP) and procalcitonin (PCT) are still widely used inflammatory markers in the clinical evaluation of sepsis [4, 5]. CRP is a non-specific inflammatory marker; thus, it can be elevated in various acute and chronic diseases (e.g. autoimmune disorders, trauma, malignancies) besides sepsis [6-8]. However, despite CRP being a general inflammatory marker, its mostly inversely proportional relation to albumin – namely the CRP:albumin ratio – has already been investigated in numerous clinical conditions (e.g. sepsis, pancreatitis, coronary artery disease, malignancies) [9-11]. CRP reaches its peak levels 48 hours after the start of the inflammatory process and significantly elevated CRP levels also have a moderate correlation with the severity of sepsis [6, 12]. Compared to CRP, PCT concentrations increase 4-6 hours after the onset of infection while PCT also showed better performance regarding the diagnosis and mortality prediction of sepsis [8, 13, 14]. However, exclusively fungal or viral infections do not elevate PCT, yet other inflammatory conditions (e.g. extensive tissue injury, pancreatitis) besides infection could result in slightly increased PCT levels [14, 15]. Furthermore, the antagonistic changes in PCT and albumin were also investigated in sepsis-related AKI [16].

Besides hs-CRP and PCT, more than 200 novel (mostly serum) sepsis biomarkers have been evaluated, yet no single marker was sensitive or specific enough for accurately diagnosing sepsis [17, 18]. However, a multi-marker approach including various promising sepsis biomarkers (e.g. presepsin, IL-6) may provide useful information regarding the early diagnosis of sepsis.

Presepsin

Presepsin (PSEP) is the soluble N-terminal fragment (MW=13 kDa) of the cluster of differentiation (CD) marker protein CD14 (MW=55 kDa), which is the receptor for lipopolysaccharide (LPS) and LPS-binding protein complexes [19, 20]. Compared to PCT, PSEP has an even more rapid response time of 2-4 hours after the onset of infection, while PSEP was also deemed valuable for the early diagnosis and prognosis of sepsis in contrast to other conditions (e.g. trauma, burn injury, major surgical operations) [21, 22]. PSEP has varying diagnostic cut-off values among 400-600 pg/mL for sepsis, yet there is a concern that PSEP concentrations are affected by kidney function [23-25]. It is presumed that PSEP is filtered by the glomeruli, then reabsorbed and catabolized within proximal tubular cells. Several studies reported increasing PSEP levels as kidney function decreases (e.g. during chronic kidney disease or sepsis-related AKI) [26-28]. However, PSEP – along with hs-CRP and PCT – could be removed from the circulation

using different modalities of renal replacement therapy (RRT), therefore potentially causing falsely low inflammatory marker levels [28, 29].

CONVENTIONAL MARKERS OF SEPSIS-RELATED AKI

AKI refers to an abrupt decrease in kidney function resulting in the retention of numerous waste products and the dysregulation of extracellular volume [30, 31]. The Kidney Disease Improving Global Outcomes (KDIGO) classification is widely used for the diagnosis of AKI based on the increase in serum creatinine (sCr) levels and the decrease of urine output, both of which are kidney function markers [32]. Urine output often decreases before the elevation of sCr concentration could be detected, yet not all reductions in urine output indicate AKI. Unfortunately, due to the non-linear relationship between glomerular filtration rate (GFR) and sCr, the increase of creatinine could be overlooked in the early phase of AKI. Therefore, the changes in these parameters do not reflect the extent of kidney damage, hence more sensitive and specific laboratory biomarkers are needed for the early diagnosis and prediction of AKI [33, 34].

So far, several novel biomarkers have been investigated to improve early diagnosis and prognosis of sepsis-related AKI [34, 35]. However, the clinical use of the studied biomarkers remains unclear due to heterogeneity of AKI itself and the limitations of novel AKI biomarkers. Regarding this, several biomarkers are discussed including tissue inhibitor of metalloproteinases-2 (TIMP-2), insulin-like growth factor-binding protein 7 (IGFBP7), neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1) and Cystatin C (CysC) while urinary actin and the most important proteins of the actin scavenger system (gelsolin, Gc-globulin) are also presented in this review.

NOVEL DAMAGE BIOMARKERS IN SEPSIS-RELATED AKI

Pre-injury phase stress markers

It has been proposed that the development of AKI is mostly preceded by a so-called acute kidney stress (AKS) phase which can occur due to several sources of renal insults (e.g. hypoperfusion, nephrotoxic drugs, cytokines, reactive oxygen species). The expression of several cell growth regulating proteins including TIMP-2 (MW=21 kDa) and IGFBP7 (MW=29 kDa) could be upregulated in the tubular system as a consequence of kidney stress, thus leading to G1 cell cycle arrest through the induction of several apoptotic pathways. In the case of prolonged kidney stress, the persistent urinary increase of these biomarkers could also indicate apoptotic tubular injury, thereby signaling the early development of AKI. Urine measurements of TIMP-2 and IGFBP7 were found useful in the early diagnosis and prognosis of AKI according to several multicentric studies. However, these biomarkers did not prove to be beneficial in patients with more severe stages of AKI while false positive results could also occur frequently in low-risk patients [36-38].

Tubular markers of AKI progression

NGAL is a glycoprotein (MW=25 kDa) of the lipid carrier protein superfamily expressed mostly on the surface of neutrophils while also being scarcely present in other cell types (e.g. nephrocytes, hepatocytes). NGAL is filtered through the glomeruli and reabsorbed in the proximal tubules under normal circumstances. NGAL expression is rapidly upregulated if the tubular system is affected during ischemic or nephrotoxic renal injury, hence NGAL is detectable in the urine as early as 3 hours after the onset of kidney damage. Plasma NGAL levels seem to have a stronger correlation with absolute neutrophil count than proinflammatory cytokines,

therefore lower plasma NGAL levels could still occur due to neutropenia in patients with systemic inflammation [39]. However, urine NGAL levels may also be significantly increased in AKI patients with neutropenia if the tubular system is affected. As NGAL also has an antimicrobial effect by binding siderophores, it was also found valuable along with hepcidin – a main regulator of iron homeostasis – in the prognosis of sepsis-related AKI. All in all, urinary NGAL elevation could reflect the decreased reabsorption of filtered NGAL due to the dysfunction or injury of the proximal tubules while its expression could also be upregulated in the tubular system during sepsis-related AKI [40-43].

KIM-1 is a transmembrane glycoprotein (MW= 39 kDa) containing extracellular immunoglobulin and mucin domains having a low expression in the kidney under physiological conditions. However, it is upregulated after ischemia-reperfusion injury, especially in proliferating de-differentiated proximal tubular epithelial cells 48 hours after injury. KIM-1 appears to be a highly sensitive marker of AKI in non-cardiac surgical patients as well as after cardiac surgery. Persistent serum KIM-1 elevation indicates ongoing tubular injury, potentially increasing the risk for the development of chronic kidney disease, while urinary KIM-1 also shows a similar correlation to kidney injury [44-47].

CysC (MW=13 kDa) is constantly produced by all nucleated cells, filtered by the glomeruli, then mostly reabsorbed and catabolized in the proximal tubular cells. The measurement of serum CysC was found to be preferable to sCr in patients after non-traumatic and traumatic amputation while also being superior in predicting cardiovascular events and mortality in elderly patients [48-50]. However, as CysC is a more reliable kidney function marker compared with sCr, it seems to be influenced by old age, large doses of corticosteroids, conditions affecting the thyroid gland, inflammation and malignancies. As the changes in sCr concentrations have some limitations, especially late in the clinical course of ICU patients, urine CysC seems to be unaffected by several non-renal factors affecting creatinine levels in sepsis-related AKI [50-53].

RELEVANCE OF ACTIN AND THE ACTIN SCAVENGER SYSTEM

Actin

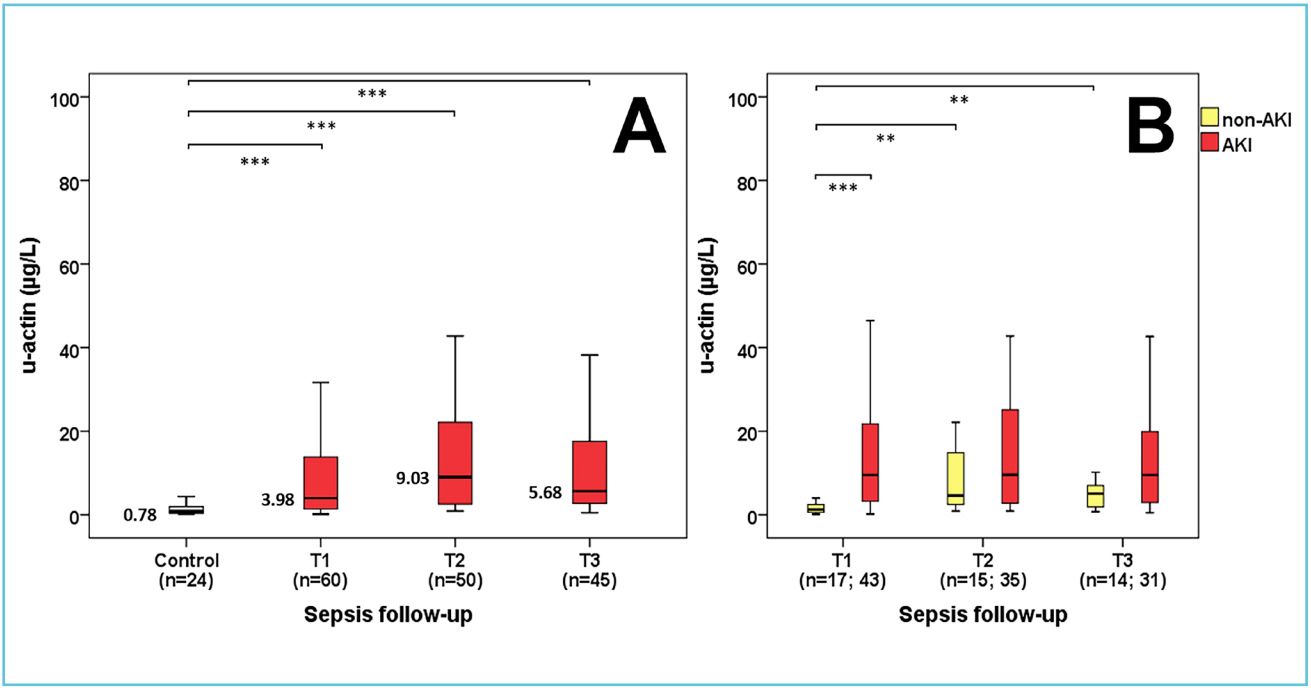
Actin is a multifunctional globular protein (MW=42 kDa) existing in monomeric/globular (G-actin) and in polymeric/filamentous (F-actin) forms. In acute tissue injuries the released extracellular actin is found to be highly toxic in the circulation due to its spontaneous polymerizing tendency causing unfavorable effects on the coagulation system. Gelsolin and Gc-globulin are the most important proteins of the so-called actin scavenger system which is responsible for binding and depolymerizing extracellular actin in the circulation, thus making the urinary appearance of these protein complexes unlikely [54-57]. However, an earlier study indicates that actin could be detected in the urine of kidney transplant patients with sustained AKI [58]. Recently published data suggest that urinary actin (u-actin) could be a complementary diagnostic biomarker to sCr in sepsis-related AKI while higher u-actin levels also seemed to reflect the severity of AKI [59]. Significantly different admission u-actin levels were found between 24 control and 60 septic patients (median: 0.78 vs. 3.98 µg/L, $p<0.001$), while samples from 17 septic non-AKI and 43 sepsis-related AKI patients also showed differences (median: 1.27 vs. 9.52 µg/L, $p<0.001$) (Fig 1). Admission u-actin levels were even more elevated in 43 patients with sepsis-related AKI and were in good agreement with the severity of AKI stages (median: 3.16 vs. 10.78 vs. 11.55 µg/L, $p<0.05$) (Fig 2). This tendency remained the same when referring u-actin to urine creatinine. Parameters of first-day

septic patient samples could discriminate AKI from non-AKI state (AUC ROC, p<0.001): u-actin: 0.876; se-creatinine: 0.875. Derived cut-off value for u-actin was 2.63 µg/L (sensitivity: 86.0%, specificity: 82.4%). Although this study has several limitations, u-actin showed moderate correlation with other urinary parameters. Furthermore, extremely elevated u-actin levels were found in sepsis-related AKI patients with RRT requirement. Despite actin being bound to the actin scavenger proteins (gelsolin, Gc-globulin) in the circulation, u-actin could appear in the urine due to both severe glomerular and/or tubular injury, so it seems that the elevation of u-actin indicates severe kidney injury (Figure 1, Figure 2).

Gelsolin

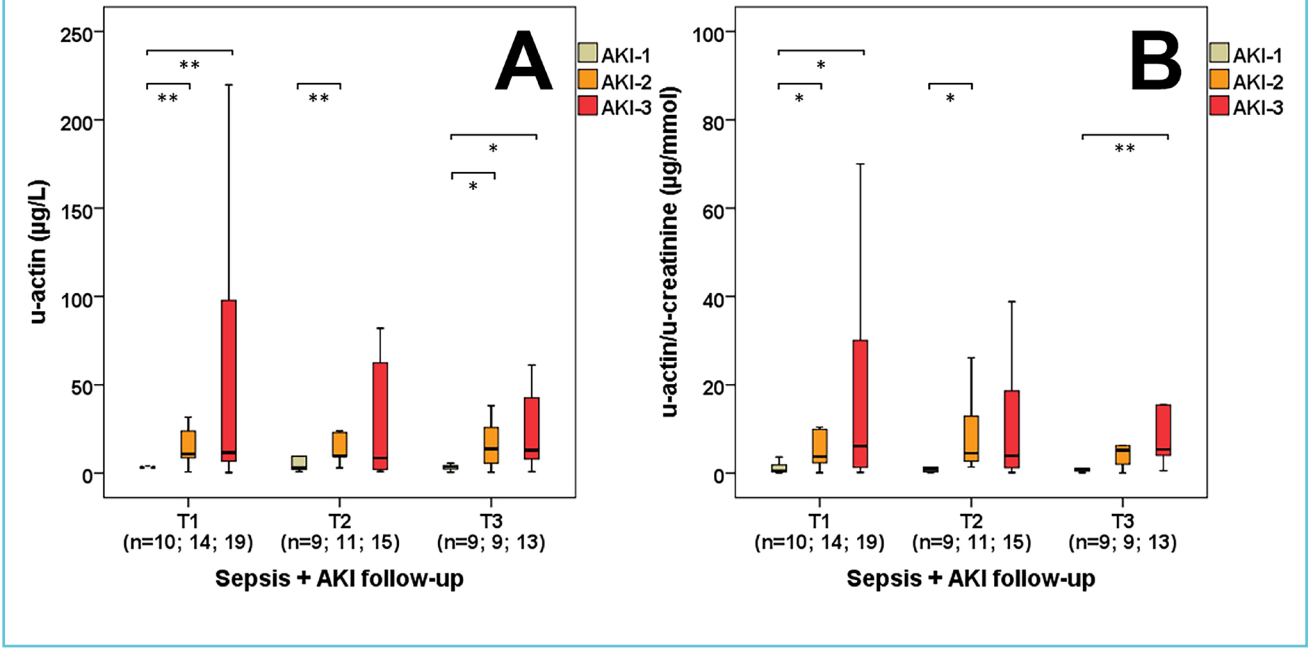
Gelsolin (GSN) is a multifunctional protein existing in three different isoforms. Secreted or plasma GSN (MW=83 kDa) is an essential component of the extracellular actin scavenger system [55, 60, 61]. Besides actin, GSN may also bind to pro-inflammatory mediators and bacterial wall components (lipoteichoic acid, LPS). Since GSN seems to have a protective role in the body (e.g. by depolymerizing actin filaments in the circulation), a growing body of evidence indicates decreasing GSN levels in various clinical conditions (e.g. severe sepsis, multiple organ dysfunction syndrome (MODS), extensive trauma, acute liver failure, myocardial infarction) [62-64]. Our previous study showed as well that the increase of serum actin was inversely proportional to the amounts of serum GSN which was associated with increased mortality rate [65]. Furthermore, the simultaneous measurement of other inflammatory parameters and GSN levels may provide valuable information in the management of critically ill patients.

Figure 1 **Urinary actin in sepsis. U-actin levels of control and septic patients (A) along with sepsis and sepsis-related AKI patients (B) during follow-up**



*Time points: T1: within 24h after admission; T2: second day morning; T3: third day morning; n: sample count. **p<0.01; ***p<0.001. Reprinted with permission from Ragán et al. (2021) (CC BY 4.0) [59].*

Figure 2 U-actin (A) and u-actin/u-creatinine (B) levels of the individual sepsis-related AKI stages during follow-up

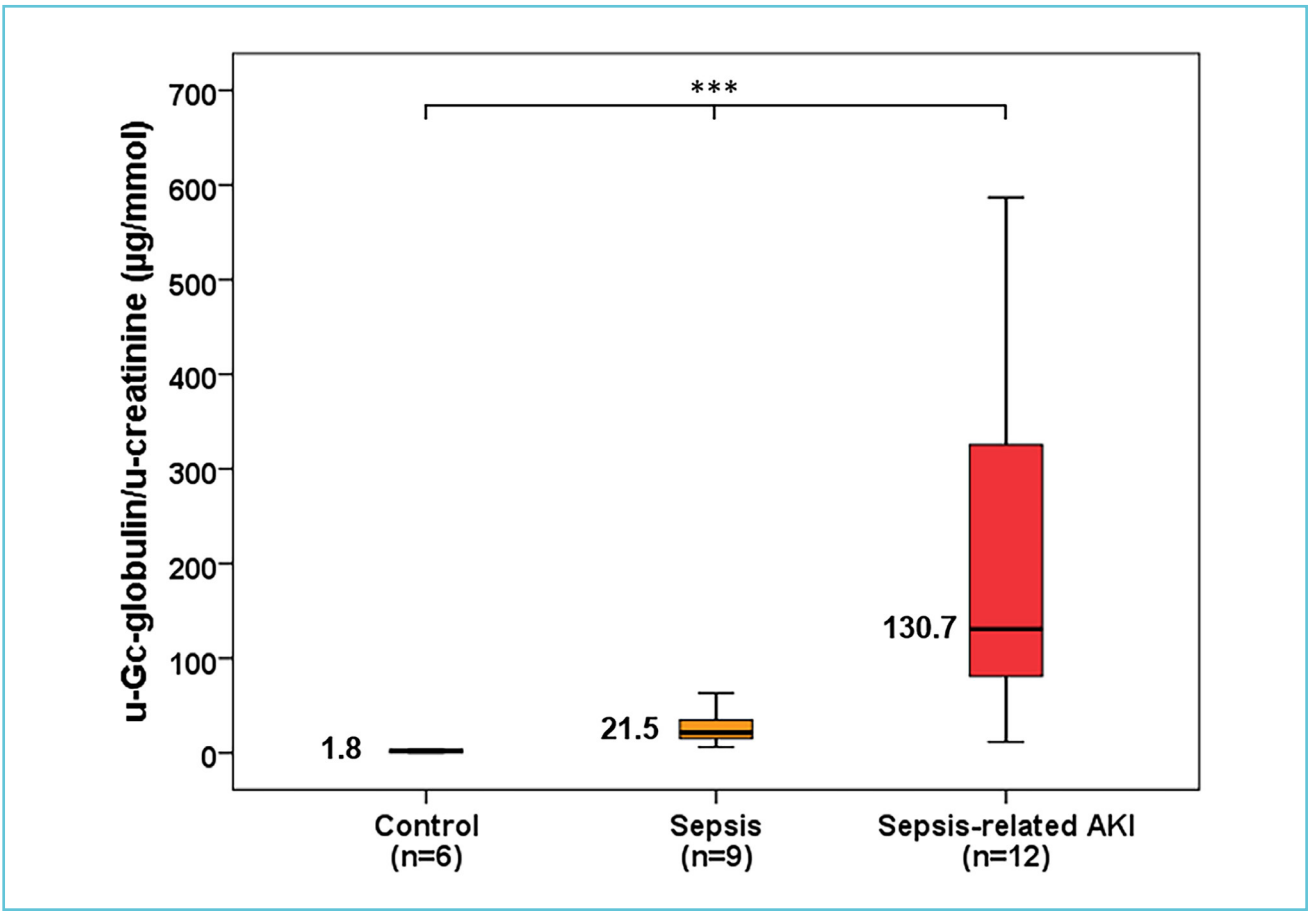


*Time points: T1: within 24h after admission; T2: second day morning; T3: third day morning; n: sample count. *p<0.05; **p<0.01. Reprinted with permission from Ragán et al. (2021) (CC BY 4.0) [59].*

Gc-globulin

Gc-globulin (MW=52-59 kDa) has 3 major isoforms (Gc1f, Gc1s, Gc2) while also being a member of the albuminoid superfamily, which consists of several transport proteins in the circulation including albumin, alpha-fetoprotein and afamin [66]. Albumin is the most abundant human serum protein acting as a transporter of endogenous and exogenous substances including thyroxine, fatty acids and drugs. Gc-globulin is mainly produced by the liver with a reference range of 300-600 mg/L, yet severely decreased levels of 50-120 mg/L were observed in acute injury or sepsis. Gc-globulin is involved in the vitamin D biosynthesis process by binding and transporting vitamin D metabolites while it also plays an important role in modulating cells of the adaptive immune response [67, 68]. Under physiological conditions, Gc-globulin is filtered freely through the glomeruli, then reabsorbed and catabolized by proximal tubular epithelial cells resulting only in a trace urinary excretion. Therefore, tubular kidney damage is expected to result in increased urinary Gc-globulin concentrations [69, 70]. There is only scarce data on urinary appearance of Gc-globulin in sepsis, yet this marker was already investigated in other conditions including endometriosis, diabetic nephropathy and contrast-induced nephropathy as well [67, 71]. Recently, our research group started investigating urinary Gc-globulin/urine creatinine (u-Gc-globulin/u-Cr) levels in sepsis-related AKI by conducting a small sample size pilot study. Compared to 6 control patients, significantly elevated admission u-Gc-globulin/u-Cr levels were found in 9 septic and 12 sepsis-related AKI patients (median: 1.8 vs. 21.5 vs. 136.7 µg/mmol, p<0.001) (Figure 3).

Figure 3 **Urinary Gc-globulin in sepsis and in sepsis-related AKI. Admission u-Gc-globulin/u-creatinine levels of control, septic and sepsis-related AKI patients.**



*n: sample count. AKI: acute kidney injury. ***p<0.001.*

SUMMARY OF NOVEL BIOMARKERS IN SEPSIS-RELATED AKI

Despite their limitations and the heterogeneity of AKI itself, the discussed laboratory markers yield valuable information regarding the early diagnosis and effective prognosis of sepsis-related AKI. Most widely known laboratory markers including TIMP-2xIGFBP-7, NGAL, KIM-1 and Cystatin C can be measured using commercially available diagnostic assays, thereby providing accurate results with a short turnaround time (less than 1 hour). Serum GSN was measured using an automated immune turbidimetric assay developed in our laboratory with a short turnaround time (less than 1 hour) as well, yet this measurement method is not yet commercially available.

However, the measurement of u-actin and u-Gc-globulin/u-creatinine was carried out using quantitative Western blot techniques, hence the routine clinical utility of these biomarkers is questionable. Therefore, our research group is currently working on the development of more rapid and efficient ELISA methods for measuring u-actin and u-Gc-globulin/u-creatinine. All of the discussed laboratory markers are shown in Table 1.

Table 1 Classification of novel AKI biomarkers

	Diagnostic utility based on renal injury site	Measurement method
Novel sepsis-related AKI biomarkers		
Urine TIMP-2xIGFBP-7 (MW=21 kDa)x(MW=29 kDa)	Tubular injury	Point of Care test
Urine NGAL (MW=25 kDa)	Tubular injury	Automated immune turbidimetric assay
Urine KIM-1 (MW=39 kDa)	Tubular injury	ELISA
Urine Cystatin C (MW=13 kDa)	Tubular injury	Automated immune turbidimetric assay
Actin scavenger system proteins		
Urinary actin (MW=42 kDa)	Glomerular and tubular injury	Western blot
Serum Gelsolin (MW=83 kDa)	Glomerular injury	Automated immune turbidimetric assay
Urine Gc-globulin (MW=52-59 kDa)	Glomerular and tubular injury	Western blot

Abbreviations: TIMP-2: tissue inhibitor of metalloproteinases-2; IGFBP7: insulin-like growth factor-binding protein 7; NGAL: neutrophil gelatinase-associated lipocalin; KIM-1: kidney injury molecule-1; MW: molecular weight; ELISA: enzyme-linked immunosorbent assay.

CONCLUSION

The early diagnosis and effective therapy of sepsis and sepsis-related AKI are essential for a successful recovery. However, the currently used biomarkers (sepsis: PCT, hs-CRP; AKI: se-creatinine, urine output) show several limitations regarding the diagnosis and prognosis of sepsis and AKI, hence investigating novel laboratory markers may prove to be beneficial in achieving a more favorable outcome. Most of the discussed AKI biomarkers provide valuable information regarding the injury of the tubular system, yet the monitoring of serum and urinary actin levels along with measuring the proteins of the actin-scavenger system (GSN, Gc-globulin) could yield more complex information for the assessment of overall renal damage involving both glomerular and tubular sources of kidney injury. Moreover, the increase of these markers could yield valuable information regarding the need for the early initiation of RRT, thus potentially attenuating renal injury and improving the outcome of sepsis-related AKI. Urinary markers provide a non-invasive tool for monitoring of inflammatory conditions. Since sepsis-related AKI is a heterogeneous clinical syndrome, it seems that the measurement of a single marker alone would be insufficient for accurate diagnostic and monitoring purposes. Therefore, a

multi-marker approach involving various serum and urinary biomarkers and the complex evaluation of the clinical parameters should improve patient management at the ICU.

❖❖❖❖❖

Acknowledgements

The present research was funded by the 2020-4.1.1.-TKP2020 project (Thematic Excellence Program 2020 - National Excellence Subprogram of the Hungarian Ministry for Innovation and Technology) and partially by the Human Reproduction National Laboratory as part of the "Establishment of National Laboratories 2020" program. Zoltán Horváth-Szalai was supported by the University of Pécs, Medical School, Hungary grant (KA-2019-36). We express our special thanks for the invaluable help of the nurses and the colleagues from every participating institute.

Ethical statement

The authors state that they have obtained institutional review board approval from the Regional Research Ethics Committee of the University of Pécs (no. 4327.316-2900/KK15/2011) conforming to the 7th revision of the Helsinki Declarations (2013) for the research described. Verbal and written informed consent were obtained from the patients for the inclusion of their medical and treatment history within this work.

Competing interests: The authors have declared that no competing interests exist.

❖❖❖❖❖

REFERENCES

1. Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, et al. Global, regional, and national sepsis incidence and mortality, 1990-2017: Analysis for the Global Burden of Disease Study. Lancet. 2020;395(10219):200-11.

2. Rhee C, Dantes R, Epstein L, Murphy DJ, Seymour CW, Iwashyna TJ, et al. Incidence and Trends of Sepsis in US Hospitals Using Clinical vs Claims Data, 2009-2014. Jama. 2017;318(13):1241-9.

3. Rhee C, Jones TM, Hamad Y, Pande A, Varon J, O'Brien C, et al. Prevalence, Underlying Causes, and Preventability of Sepsis-Associated Mortality in US Acute Care Hospitals. Jama Network Open. 2019;2(2):e187571.

4. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). Jama. 2016;315(8):801-10.

5. Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, Ferrer R, et al. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. Intensive Care Medicine. 2017;43(3):304-77.

6. Póvoa P, Almeida E, Moreira P, Fernandes A, Mealha R, Aragão A, et al. C-reactive protein as an indicator of sepsis. Intensive Care Medicine. 1998;24(10):1052-6.

7. Wang HE, Shapiro NI, Safford MM, Griffin R, Judd S, Rodgers JB, et al. High-sensitivity C-reactive protein and risk of sepsis. PloS One. 2013;8(7):e69232.

8. Tan M, Lu Y, Jiang H, Zhang L. The diagnostic accuracy of procalcitonin and C-reactive protein for sepsis: A systematic review and meta-analysis. Journal of Cellular Biochemistry. 2019;120(4):5852-9.

9. Kaplan M, Ates I, Akpinar MY, Yuksel M, Kuzu UB, Kacar S, et al. Predictive value of C-reactive protein/albumin ratio in acute pancreatitis. Hepatobiliary & Pancreatic Diseases International: HBPD INT. 2017;16(4):424-30.

10. Karabag Y, Cagdas M, Rencuzogullari I, Karakoyun S, Artac I, Ilis D, et al. Relationship between C-reactive protein/albumin ratio and coronary artery disease severity in patients with stable angina pectoris. Journal of Clinical Laboratory Analysis. 2018;32(7):e22457.

11. Miyamoto T, Fujitani M, Fukuyama H, Hatanaka S, Koizumi Y, Kawabata A. The C-Reactive Protein/Albumin Ratio Is Useful for Predicting Short-Term Survival in Cancer and Noncancer Patients. Journal of Palliative Medicine. 2019;22(5):532-7.

12. Sierra R, Rello J, Bailén MA, Benítez E, Gordillo A, León C, et al. C-reactive protein used as an early indicator of infection in patients with systemic inflammatory response syndrome. Intensive Care Medicine. 2004;30(11):2038-45.

13. Meisner M. Pathobiochemistry and clinical use of procalcitonin. Clinica Chimica Acta. 2002;323(1):17-29.

14. Wacker C, Prkno A, Brunkhorst FM, Schlattmann P. Procalcitonin as a diagnostic marker for sepsis: a systematic

review and meta-analysis. The Lancet Infectious Diseases. 2013;13(5):426-35.

15. Uzzan B, Cohen R, Nicolas P, Cucherat M, Perret G-Y. Procalcitonin as a diagnostic test for sepsis in critically ill adults and after surgery or trauma: A systematic review and meta-analysis. Critical Care Medicine. 2006;34(7): 1996-2003.

16. Chen L, Wu X, Qin H, Zhu H. The PCT to Albumin Ratio Predicts Mortality in Patients With Acute Kidney Injury Caused by Abdominal Infection-Evoked Sepsis. Frontiers in Nutrition. 2021;8(179).

17. Giannakopoulos K, Hoffmann U, Ansari U, Bertsch T, Borggrefe M, Akin I, et al. The Use of Biomarkers in Sepsis: A Systematic Review. Current Pharmaceutical Biotechnology. 2017;18(6):499-507.

18. Pierrakos C, Velissaris D, Bisdorff M, Marshall JC, Vincent JL. Biomarkers of sepsis: time for a reappraisal. Critical Care. 2020;24(1):287.

19. Shirakawa K, Naitou K, Hirose J, Takahashi T, Furusako S. Presepsin (sCD14-ST): development and evaluation of one-step ELISA with a new standard that is similar to the form of presepsin in septic patients. Clinical Chemistry and Laboratory Medicine. 2011;49(5):937-9.

20. Yaegashi Y, Shirakawa K, Sato N, Suzuki Y, Kojika M, Imai S, et al. Evaluation of a newly identified soluble CD14 subtype as a marker for sepsis. Journal of Infection and Chemotherapy. 2005;11(5):234-8.

21. Endo S, Suzuki Y, Takahashi G, Shozushima T, Ishikura H, Murai A, et al. Usefulness of presepsin in the diagnosis of sepsis in a multicenter prospective study. Journal of Infection and Chemotherapy. 2012;18(6):891-7.

22. Shozushima T, Takahashi G, Matsumoto N, Kojika M, Okamura Y, Endo S. Usefulness of presepsin (sCD14-ST) measurements as a marker for the diagnosis and severity of sepsis that satisfied diagnostic criteria of systemic inflammatory response syndrome. Journal of Infection and Chemotherapy. 2011;17(6):764-9.

23. Velissaris D, Zareifopoulos N, Karamouzos V, Karanikolas E, Pierrakos C, Koniari I, et al. Presepsin as a Diagnostic and Prognostic Biomarker in Sepsis. Cureus. 2021;13(5):e15019.

24. Wu CC, Lan HM, Han ST, Chaou CH, Yeh CF, Liu SH, et al. Comparison of diagnostic accuracy in sepsis between presepsin, procalcitonin, and C-reactive protein: a systematic review and meta-analysis. Annals of Intensive Care. 2017;7(1):91.

25. Zhang X, Liu D, Liu Y-N, Wang R, Xie L-X. The accuracy of presepsin (sCD14-ST) for the diagnosis of sepsis in adults: a meta-analysis. Critical Care. 2015;19(1):323.

26. Nagata T, Yasuda Y, Ando M, Abe T, Katsuno T, Kato S, et al. Clinical impact of kidney function on presepsin levels. PloS One. 2015;10(6):e0129159.

27. Nakamura Y, Hoshino K, Kiyomi F, Kawano Y, Mizunuma M, Tanaka J, et al. Comparison of accuracy of presepsin and procalcitonin concentrations in diagnosing sepsis in patients with and without acute kidney injury. Clinica Chimica Acta. 2019;490:200-6.

28. Shimoyama Y, Umegaki O, Kadono N, Minami T. Presepsin and prognostic nutritional index are predictors of septic acute kidney injury, renal replacement therapy initiation in sepsis patients, and prognosis in septic acute kidney injury patients: a pilot study. BMC Nephrology. 2021;22(1):219.

29. Honore PM, Jacobs R, Hendrickx I, De Waele E, Van Gorp V, Spapen HD. Presepsin and sepsis-induced acute kidney injury treated with continuous renal replacement therapy: will another promising biomarker bite the dust? Critical Care. 2015;19:428.

30. Uchino S, Kellum JA, Bellomo R, Doig GS, Morimatsu H, Morgera S, et al. Acute renal failure in critically ill patients: a multinational, multicenter study. Jama. 2005;294(7): 813-8.

31. Hoste EAJ, Kellum JA, Selby NM, Zarbock A, Palevsky PM, Bagshaw SM, et al. Global epidemiology and outcomes of acute kidney injury. Nature Reviews Nephrology. 2018;14(10):607-25.

32. Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group. KDIGO Clinical Practice Guideline for Acute Kidney Injury. Kidney International Supplements. 2012;2(1):1-138.

33. Waikar SS, Betensky RA, Bonventre JV. Creatinine as the gold standard for kidney injury biomarker studies? Nephrology, Dialysis, Transplantation. 2009;24(11):3263-5.

34. Oh DJ. A long journey for acute kidney injury biomarkers. Renal Failure. 2020;42(1):154-65.

35. Bellomo R, Kellum JA, Ronco C, Wald R, Martensson J, Maiden M, et al. Acute kidney injury in sepsis. Intensive Care Medicine. 2017;43(6):816-28.

36. Kashani K, Al-Khafaji A, Ardiles T, Artigas A, Bagshaw SM, Bell M, et al. Discovery and validation of cell cycle arrest biomarkers in human acute kidney injury. Critical Care. 2013;17(1):R25.

37. Su Y, Gong Z, Wu Y, Tian Y, Liao X. Diagnostic Value of Urine Tissue Inhibitor of Metalloproteinase-2 and Insulin-Like Growth Factor-Binding Protein 7 for Acute Kidney Injury: A Meta-Analysis. PloS One. 2017;12(1):e0170214.

38. Kellum JA, Artigas A, Gunnerson KJ, Honore PM, Kampf JP, Kwan T, et al. Use of Biomarkers to Identify Acute Kidney

Injury to Help Detect Sepsis in Patients With Infection. Critical Care Medicine. 2021;49(4):e360-e8.

39. Choi JW, Fujii T. The Prevalence of Low Plasma Neutrophil Gelatinase-Associated Lipocalin Level in Systemic Inflammation and its Relationship with Proinflammatory Cytokines, Procalcitonin, Nutritional Status, and Leukocyte Profiles. Clinical Laboratory. 2019;65(6).

40. Wang K, Xie S, Xiao K, Yan P, He W, Xie L. Biomarkers of Sepsis-Induced Acute Kidney Injury. BioMed Research International. 2018;2018:6937947.

41. Albert C, Zapf A, Haase M, Rover C, Pickering JW, Albert A, et al. Neutrophil Gelatinase-Associated Lipocalin Measured on Clinical Laboratory Platforms for the Prediction of Acute Kidney Injury and the Associated Need for Dialysis Therapy: A Systematic Review and Meta-analysis. American Journal of Kidney Diseases. 2020;76(6):826-41 e1.

42. Chen JJ, Lee TH, Lee CC, Chang CH. Using lipocalin as a prognostic biomarker in acute kidney injury. Expert Review of Molecular Diagnostics. 2021;21(5):455-64.

43. Qiu Z-L, Yan B-Q, Zhao R, Xu D-W, Shen K, Deng X-q, et al. Combination of hepcidin with neutrophil gelatinase-associated lipocalin for prediction of the development of sepsis-induced acute kidney injury. Clinica Chimica Acta. 2021;523:38-44.

44. Han WK, Bailly V, Abichandani R, Thadhani R, Bonventre JV. Kidney Injury Molecule-1 (KIM-1): A novel biomarker for human renal proximal tubule injury. Kidney International. 2002;62(1):237-44.

45. Ichimura T, Hung CC, Yang SA, Stevens JL, Bonventre JV. Kidney injury molecule-1: a tissue and urinary biomarker for nephrotoxicant-induced renal injury. American Journal of Physiology Renal physiology. 2004;286(3):F552-63.

46. Huang Y, Don-Wauchope AC. The clinical utility of kidney injury molecule 1 in the prediction, diagnosis and prognosis of acute kidney injury: a systematic review. Inflammation & Allergy Drug Targets. 2011;10(4):260-71.

47. Shao X, Tian L, Xu W, Zhang Z, Wang C, Qi C, et al. Diagnostic Value of Urinary Kidney Injury Molecule 1 for Acute Kidney Injury: A Meta-Analysis. PloS One. 2014;9(1): e84131.

48. Thurlow JS, Abbott KC, Linberg A, Little D, Fenderson J, Olson SW. SCr and SCysC Concentrations Before and After Traumatic Amputation in Male Soldiers: A Case-Control Study. American Journal of Kidney Diseases. 2014;63(1): 167-70.

49. Aakjær M, Houlind MB, Treldal C, Ankarfeldt MZ, S Jensen P, Andersen O, et al. Differences in Kidney Function Estimates Based on Creatinine and/or Cystatin C in Non-Traumatic Amputation Patients and Their Impact on Drug Prescribing. Journal of Clinical Medicine. 2019;8(1):89.

50. Shlipak MG, Sarnak MJ, Katz R, Fried LF, Seliger SL, Newman AB, et al. Cystatin C and the Risk of Death and Cardiovascular Events among Elderly Persons. New England Journal of Medicine. 2005;352(20):2049-60.

51. Séronie-Vivien S, Delanaye P, Piéroni L, Mariat C, Froissart M, Cristol JP. Cystatin C: current position and future prospects. Clinical Chemistry and Laboratory Medicine. 2008;46(12):1664-86.

52. Martensson J, Martling CR, Oldner A, Bell M. Impact of sepsis on levels of plasma cystatin C in AKI and non-AKI patients. Nephrology, Dialysis, Transplantation. 2012;27(2): 576-81.

53. Szirmay B, Kustan P, Horvath-Szalai Z, Ludany A, Lakatos A, Muhl D, et al. Novel automated immune turbidimetric assay for routine urinary cystatin-C determinations. Bioanalysis. 2018;10(6):377-84.

54. Haddad JG, Harper KD, Guoth M, Pietra GG, Sanger JW. Angiopathic consequences of saturating the plasma scavenger system for actin. Proceedings of the National Academy of Sciences of the United States of America. 1990; 87(4):1381-5.

55. Lee WM, Galbraith RM. The extracellular actin-scavenger system and actin toxicity. The New England Journal of Medicine. 1992;326(20):1335-41.

56. Belsky JB, Morris DC, Bouchebl R, Filbin MR, Bobbitt KR, Jaehne AK, et al. Plasma levels of F-actin and F:G-actin ratio as potential new biomarkers in patients with septic shock. Biomarkers: Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals. 2016;21(2):180-5.

57. Sudakov NP, Klimenkov IV, Byvaltsev VA, Nikiforov SB, Konstantinov YM. Extracellular Actin in Health and Disease. Biochemistry Biokhimiia. 2017;82(1):1-12.

58. Kwon O, Molitoris BA, Pescovitz M, Kelly KJ. Urinary actin, interleukin-6, and interleukin-8 may predict sustained ARF after ischemic injury in renal allografts. American Journal of Kidney Diseases. 2003;41(5):1074-87.

59. Ragan D, Kustan P, Horvath-Szalai Z, Szirmay B, Bugyi B, Ludany A, et al. Urinary actin, as a potential marker of sepsis-related acute kidney injury: A pilot study. PloS One. 2021; 16(7):e0255266.

60. Maiti S, Bamburg JR. Actin-Capping and -Severing Proteins. In: Lennarz WJ, Lane MD, editors. Encyclopedia of Biological Chemistry (Second Edition). Waltham: Academic Press; 2013. p. 18-26.

61. Krebs J. Calcium | Calcium-Binding Proteins: Cytosolic (Annexins, Gelsolins, and C2-Domain Proteins)☆. In: Jez J, editor. Encyclopedia of Biological Chemistry III (Third Edition). Oxford: Elsevier; 2021. p. 621-9.

62. Suhler E, Lin W, Yin HL, Lee WM. Decreased plasma gelsolin concentrations in acute liver failure, myocardial

infarction, septic shock, and myonecrosis. Critical Care Medicine. 1997;25(4):594-8.

63. Dahl B, Schiodt FV, Ott P, Gvozdenovic R, Yin HL, Lee WM. Plasma gelsolin is reduced in trauma patients. Shock. 1999;12(2):102-4.

64. Lee PS, Patel SR, Christiani DC, Bajwa E, Stossel TP, Waxman AB. Plasma gelsolin depletion and circulating actin in sepsis: a pilot study. PloS One. 2008;3(11):e3712.

65. Horvath-Szalai Z, Kustan P, Muhl D, Ludany A, Bugyi B, Koszegi T. Antagonistic sepsis markers: Serum gelsolin and actin/gelsolin ratio. Clinical Biochemistry. 2017;50(3): 127-33.

66. Mozzi A, Forni D, Cagliani R, Pozzoli U, Vertemara J, Bresolin N, et al. Albuminoid genes: evolving at the interface of dispensability and selection. Genome Biology and Evolution. 2014;6(11):2983-97.

67. Delanghe JR, Speeckaert R, Speeckaert MM. Behind the scenes of vitamin D binding protein: more than vitamin D binding. Best Practice & Research Clinical Endocrinology & Metabolism. 2015;29(5):773-86.

68. Bouillon R, Schuit F, Antonio L, Rastinejad F. Vitamin D Binding Protein: A Historic Overview. Frontiers in Endocrinology. 2020;10(910).

69. Dahl B, Schiødt FV, Ott P, Wians F, Lee WM, Balko J, et al. Plasma concentration of Gc-globulin is associated with organ dysfunction and sepsis after injury. Critical Care Medicine. 2003;31(1):152-6.

70. Horvath-Szalai Z, Kustan P, Szirmay B, Lakatos A, Christensen PH, Huber T, et al. Predictive value of serum gelsolin and Gc globulin in sepsis - a pilot study. Clinical Chemistry and Laboratory Medicine. 2018;56(8):1373-82.

71. Chaykovska L, Heunisch F, von Einem G, Alter ML, Hocher C-F, Tsuprykov O, et al. Urinary Vitamin D Binding Protein and KIM-1 Are Potent New Biomarkers of Major Adverse Renal Events in Patients Undergoing Coronary Angiography. PloS One. 2016;11(1):e0145723.

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

This is a Platinum Open Access Journal distributed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to really understand and improve the system of internal quality control and external quality assessment in the accreditation process of the medical laboratory?

Milan Skitek[1], Flávia Martinello[2], Aleš Jerin[1]

[1] *University Medical Centre Ljubljana, Clinical Institute of Clinical Chemistry and Biochemistry, Ljubljana, Slovenia*

[2] *Department of Clinical Analysis, Federal University of Santa Catarina, Florianopolis, Brazil*

ARTICLE INFO

Corresponding author:
Milan Skitek
University Medical Centre Ljubljana,
Clinical Institute of Clinical
Chemistry and Biochemistry
Njegoševa 4, 1000 Ljubljana
Slovenia
E-mail: milan.skitek@kclj.si

Key words:
internal quality control, quality specifications, commutability, external quality assessment, measurement uncertainty

ABSTRACT

Internal quality control (IQC) regarding process to monitor analytical stability has a long tradition in laboratory medicine. The satisfactory results with different quality specifications of the IQC ensure the acceptability of the examination results. Although the statistical IQC is satisfactory some problems exist, resulting in unreliable patients' results due several reasons (non-commutable control materials, lot to lot difference of reagents, false interpreting test results regarding autovalidation or autoverification, different analytical and clinical specifications or goals etc.). Therefore, the results and findings of IQC have to be connected with external quality assessment (EQA) in order to provide the system of measurement of uncertainty (MU) with correct interpretation of laboratory result and detection relevant and significant shifts and drifts in medical laboratory.

INTRODUCTION

IQC is an important tool within the laboratory testing to assure the quality of results produced in medical laboratories. It is one of the cornerstones of the accreditation process of medical laboratories, primarily used in routine practice to monitor system performance under stable conditions and to allow analytical failures that affect performance to be detected [1,2]. The reliable tests depend on both IQC and EQA being performed. Commutability of reference and control materials in IQC and EQA is key to ensuring the quality of measurements in laboratory medicine. The International Vocabulary of Metrology (VIM) defines the commutability of a reference material (RM) as the property demonstrated by the closeness of agreement between the relation among the measurement results for a stated quantity in the material (employed as a calibrator), obtained according to two given measurement procedures, and the relation obtained among the measurement results for patient samples. In a simple way, the commutability is the ability of an RM or control material to show inter-assay properties comparable to those of human samples [3,4]. How to assess commutability has been covered in the Clinical and Laboratory Standards Institute (CLSI) guidelines and through the recommendations of the International Federation of Clinical Chemistry (IFCC) Working Group on Commutability (WG-C) [5-9].

IQC PROGRAMS

There is considerable variation in laboratory practices with regard to the review of IQC, and the literature is not exhaustive on the subject of own control limits and its interpretation. This is the main difference regarding IQC from other scientific disciplines in comparison with laboratory medicine where some questions have recently been raised about understanding of IQC [10]. Many efforts was made to stress the routine interpretation and challenges related to own results for IQC management including for the selected tumor markers and hormones which proved that the quality specifications based on biological variation best fit the analytical and clinical purpose of laboratory tests. We must be aware that the manufacturer's method specifications and control ranges should be used carefully comparing with our results on field and our own analytical goals. While the average results in IQC tended to get closer to the manufacturer value by increasing the number of measurements, the analytical coefficient of variation (CV_A) tended to increase. Most parameters showed significant differences between initial and cumulative CV_A, which were lower than the manufacturer's specifications [11].

EQA PROGRAMS

The EQA programs are optimal tools for evaluating the reliability of commercial measuring systems and the clinical suitability of measurements provided by clinical laboratories. However, EQAs must be appropriately structured. Efforts by EQA providers should be made to meet criteria allowing the evaluation of the performance of participating laboratories in terms of traceability of their measurements. This requires assigning values (and uncertainty) to control materials with reference measurement procedures, defining and applying clinically allowable performance specifications for judging the quality of results and using materials of proved commutability. Only materials with proved commutability are relevant for directly transferring of laboratory testing to the measurement of patient samples [12].

UNCERTAINTY

By quantifying the measurement uncertainty (MU) or the previously used total allowable error

(TAE), both the clinical laboratory and the physician can have an objective estimate of the results' quality. ISO 15189 declare that "laboratory shall consider MU when interpreting measured quantity values. Upon request, the laboratory shall make its estimates of MU available to laboratory users" [1]. In our opinion MU should be available with interpretation on laboratory report without any request from users.

Different approaches and formulas have been proposed how to determine the MU in medical laboratory with imprecision and bias of the methods considered as components of the MU (Nordtest, Eurolab, Cofrac etc.). The bias could be obtained from certified reference calibrators (CRC), proficiency tests (PT), and inter-laboratory internal quality control scheme (IQCS) programs. The bias uncertainty, the combined and the expanded uncertainty could be estimated using the different mentioned models or approaches. In our study the bias was highest using PT, followed by CRC and IQC data, which were similar. The Cofrac approach showed the highest uncertainties and the Eurolab approach requires additional measurements to obtain uncertainty data. In summary, the Nordtest approach using IQC data was therefore found to be the most practical formula [13-15].

COMMUTABILITY

IQC and EQA materials are frequently not assessed for commutability because of technical and economic concerns. The use of single-donor samples, which is preferable to overcome commutability problems, may however limit the achievement of adequate volumes of samples needed for preparing sufficient amount of control materials [16,17]. On the other hand, pooled samples have the potential limitation that interactions of components such as proteins may cause modification of the matrix.The European Federation of Laboratory Medicine (EFLM) has recently stressed the need that the especially EQA material matrix and its commutability should be specified by providers, because the interpretation of differences between results in an EQA program is strongly dependent on the nature of the employed material [18]. Based on the results of some projects of analytical performance in general clinical chemistry using commutable samples targeted with reference measurement procedures it's obvious that the use of commutable samples especially in EQA is mandatory to change conventional EQA using non-commutable materials and consensus 'peer' group assessment with the EQA programme based on clinically oriented analytical performance specifications that meet metrological criteria and traceability [19-21]. The commutability also matters for IQC materials that should be used by clinical laboratories to derive the random component of the uncertainty of measured results. The material evaluating the random uncertainty must be different from the control material used for checking the alignment of the measuring systems and should be commutable, closely resembling to patient sample, to provide accurate information about the imprecision performance of the assay [22-23].

DISCUSSION

We provided a brief overview of the practical importance of IQC in connection with EQA programs using commutable materials in laboratory medicine. They have to be employed either as common calibrators for implementing metrological traceability or as control materials in EQA and IQC programs within the total testing pathway. The use of non-commutable RMs may introduce a significant bias in the calibrated procedures producing incorrect results for patient samples. The non-commutable materials in EQA programs prevents the transferability of participating laboratory performance

to the measurement of patient samples. Only commutable control materials may provide the proper information for the imprecision, bias and estimation of measurement uncertainty. Providers of reference and control materials should assess the commutability of those materials before their use.

The importance of commutability has essential role in standardization and accreditation process, consistent clinical decisions and improving patient outcomes with additional use and rarely implemented of patient's test results for laboratory QC monitoring. The only exception is haematology testing where Bull's patient based real-time QC algorithm which was accepted and implemented in routine QC practices [24]. In the last two decades we have faced progress with the improved laboratory total automation, information technology and standardization/ harmonization of laboratory methods, so there are no more obstacles and limitations for such algorithms and use of "big data" in laboratory QC monitoring processes.

❖❖❖❖❖

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Ethical approval

None needed.

❖❖❖❖❖

REFERENCES

1. International Organization for Standardization (ISO). ISO 15189 Medical laboratories - Requirements for quality and competence., Geneva, Switzerland, 2012.

2. Giannoli JM, Albarede S, Avellan T, Bouilloux JP, Cartier R, Cohen R, et al. Recommendations for the application and the follow-up of quality controls in medical biology laboratories. Ann Biol Clin (Paris) 2019; 77577–97.

3. International Organization for Standardization (ISO). ISO 17034 General requirements for the competence of reference material producers, Geneva, Switzerland, 2016.

4. Franzini C, Ceriotti F. Impact of reference materials on accuracy in clinical chemistry. Clin Biochem 1998; 31: 449–57.

5. Clinical and Laboratory Standards Institute (CLSI). CLSI. Characterization and qualification of commutable reference materials for laboratory medicine; Approved guideline. Document EP30-A (formerly C53-A). Wayne, PA, 2010.

6. Clinical and Laboratory Standards Institute (CLSI). CLSI Evaluation of commutability of processed samples: approved guideline – 3rd ed. Document EP14-A3. Wayne, PA, 2014.

7. Miller WG, Schimmel H, Rej R, Greenberg N, Ceriotti F, Burns C, et al. IFCC working group recommendations for assessing commutability. Part 1: General experimental design. Clin Chem 2018; 64:447–54

8. Nilsson G, Budd JR, Greenberg N, Delatour V, Rej R, Panteghini M, et al. IFCC working group recommendations for assessing commutability. Part 2: Using the difference in bias between a reference material and clinical samples. Clin Chem 2018; 64:455–64.

9. Budd JR, Weykamp C, Rej R, MacKenzie F, Ceriotti F, Greenberg N, et al. IFCC working group recommendations for assessing commutability. Part 3: Using the calibration effectiveness of a reference material. Clin Chem 2018; 64:465–74.

10. Thompson M. Statistical internal quality control (SIQC) in chemical measurement – do we really understand it? Accred Qual Assur 2021; 26:700-3

11. Martinello F, Skitek M, Jerin A. Verifying and assigning own target values and ranges for internal quality control. Accred Qual Assur 2019; 24:305-12

12. Braga F, Pasqualetti S, Panteghini M. The role of external quality assessment in the verification of in vitro medical diagnostics in the traceability era. Clin Biochem 2018; 57:23–28

13. Martinello F, Snoj N, Skitek M, Jerin A. The top-down approach to measurement uncertainty: which formula

should we use in laboratory medicine? Biochem Medica 2020; 2:1-9.

14. Rigo-Bonnin R, Díaz-Troyano N, García-Tejada L, Marcè-Galindo A, Valbuena-Asensio M, Canalias F. Estimation of the measurement uncertainty and practical suggestion for the description of the metrological traceability in clinical laboratories. Biochem Med (Zagreb) 2021;31(1):010501; https://doi.org/10.11613/BM.2021.01050.

15. International Organization for Standardization (ISO). Medical laboratories — Practical guidance for the estimation of measurement uncertainty. ISO/TS 20914. Geneva, Switzerland: ISO, 2019.

16. Skitek M. Practical assessment of the suitability of IQC control materials in haemocytometry based on biologic goals. Accred Qual Assur 2003; 8:514-9.

17. Skitek M. Acceptability limits based on biology goals in haematology EQAS. Accred Qual Assur 2005; 10:112-5.

18. Jones GRD, Albarede S, Kesseler D, MacKenzie F, Mammen J, Ped-ersen M, et al. EFLM Task Finish Group – Analytical Performance Specifications for EQAS (TFG-APSEQA). Analytical performance specifications for external quality assessment – definitions and descriptions. Clin Chem Lab Med 2017; 55:949–55.

19. Weykamp C, Secchiero S, Plebani M, Thelen M, Cobbaert C, Thomas A, et al. Analytical performance of 17 general chemistry analytes across countries and across manufacturers in the INPUtS project of EQA organizers in Italy, the Netherlands, Portugal, United Kingdom and Spain. Clin Chem Lab Med 2017; 55:203–11.

20. International Organization for Standardization (ISO). In vitro diagnostic medical devices. Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples. ISO 17511. Geneva, Switzerland: ISO, 2020.

21. Kristensen GB, Rustad P, Berg JP, Aakre KM. Analytical bias exceeding desirable quality goal in 4 out of 5 common immunoassays: results of a native single serum sample external quality assessment program for cobalamin, folate, ferritin, thyroid-stimulating hormone, and free T4 analyses. Clin Chem 2016; 62:1255–63.

22. Skitek M. Imprecision and instability of common analytes in urine analysed by semi-automated dipstick urinalysis. Accred Qual Assur 2004; 9:700-3.

23. Hage-Sleiman M, Capdevila L, Bailleul S, Lefevre G. High-sensitivity cardiac troponin-I analytical imprecisions evaluated by internal quality control or imprecision profile. Clin Chem Lab Med 2019; 57:e49–51.

24. Skitek M. Study of stabilization mechanisms of flow hemocytometry control blood. PhD Thesis, University of Ljubljana, Faculty of Pharmacy, Ljubljana 1996.

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

This is a Platinum Open Access Journal distributed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Hematological findings in lysosomal storage disorders: a perspective from the medical laboratory

Andrés Felipe Leal[1], Wendy G. Nieto[2], Estephania Candelo[3,5,6], Harry Pachajoa[3,4,6], Carlos Javier Alméciga-Díaz[1]

[1] *Institute for the Study of Inborn Errors of Metabolism, Faculty of Science, Pontificia Universidad Javeriana, Bogotá D.C., Colombia*

[2] *Translational Biomedical Research Group, Centro de Investigaciones, Fundación Cardiovascular de Colombia, Santander, Colombia*

[3] *Department of Medical Basic Sciences, Faculty of Health Sciences, Universidad Icesi, Cali, Colombia*

[4] *Department of Medical Genetics, Fundación Valle del Lili, Cali, Colombia*

[5] *Centro de Investigaciones Clínicas, Fundación Valle del Lili, Cali, Colombia*

[6] *Congenital Abnormalities and Rare Disease Centre (CIACER), Cali, Colombia*

ARTICLE INFO

Co-corresponding authors:
Andrés Felipe Leal, B.Sc., M.Sc.
Carlos J. Alméciga-Díaz, BPharm, Ph.D.

Institute for the Study of Inborn Errors of Metabolism, Faculty of Science
Pontificia Universidad Javeriana, Cra. 7 No. 43-82
Building 54, Room 305A
Bogotá D.C., 110231
Colombia
Tel: +57-1 3208320 Ext 4140
Fax: +57-1 3208320 Ext 4099
E-mail: lealb.af@javeriana.edu.co
E-mail: cjalmeciga@javeriana.edu.co

Key words:
Alder-Reilly abnormalities, hematological alterations, leukocyte morphology, lysosomal storage disorders

ABSTRACT

Lysosomal storage disorders (LSDs) are a group of rare and genetic diseases produced by mutations in genes coding for proteins involved in lysosome functioning. Protein defect leads to the lysosomal accumulation of undegraded macromolecules including glycoproteins, glycosaminoglycans, lipids, and glycogen. Depending on the stored substrate, several pathogenic cascades may be activated leading to multisystemic and progressive disorders affecting the brain, eye, ear, lungs, heart, liver, spleen, kidney, skin, or bone. In addition, for some of these disorders, hematological findings have been also reported. In this paper, we review the major hematological alterations in LSDs based on 56 case reports published between 2010 and 2020. Hematological alterations were reported in sphingolipidosis, mucopolysaccharidoses, mucolipidoses, neuronal ceroid

lipofuscinosis, glycogenosis, glycoproteinosis, cystinosis, and cholesteryl ester storage disease. They were reported alterations in red cell linage and leukocytes, such as anemia and morphology changes in eosinophils, neutrophils, monocytes, and lymphocytes. In addition, changes in platelet counts (thrombocytopenia) and leukocyte abnormalities on non-peripheral blood samples were also reported for some LSDs. Although in most of the cases these hematological alterations are not pathognomonic of a specific disease or group of LSDs, since they can be easily identified in general clinical laboratories, their identification may contribute to the diagnosis of these disorders. In this sense, we hope that this review contributes to the awareness of the importance of hematological alterations in the diagnosis of LSDs.

❖❖❖❖❖

1. INTRODUCTION

Lysosomal storage disorders (LSDs) are a group of monogenic metabolic diseases produced by mutations in genes encoding for proteins involved in the lysosomal function. These mutations lead to the synthesis of proteins with none or reduced activity, producing the progressive accumulation of partially degraded substrates into the lysosome [1]. Although the clinical, diagnostics, and pathophysiology can be heterogeneous, the

Table 1 Classification of lysosomal storage disorders

Group of LSD (Accumulated substrate)	Disease	Inheritance	OMIM number
Glycogenoses (Glycogen)	Danon Disease	X-LD	300257
	Pompe Disease	AR	232300
Glycoproteinoses (Glycoproteins)	Aspartylglucosaminuria Disease	AR	208400
	Fucosidosis	AR	230000
	Galactosialidosis	AR	256540
	α-Mannosidosis	AR	248500
	β-Mannosidosis	AR	248510
	Schindler Disease	AR	609241
	Sialidosis	AR	256550
Mucolipidoses (Mucolipids)	Type I-IV	AR	252500
Mucopolysaccharidoses (Glycosaminoglycans)	Type I (Hurler Syndrome)	AR	607014

	Type II (Hunter Syndrome)	X-LR	309900
	Type III (Sanfilippo Syndrome)	AR	252920
	Type IV (Morquio Syndrome)	AR	253000
	Type VI (Maroteaux -Lamy Syndrome)	AD	184095
	Type VII (Sly Syndrome)	AR	253220
	Type IX (Natowicz Syndrome)	AR	601492
	*MPS-Plus syndrome	AR	617303
Neuronal ceroid lipofuscinoses (Lipofuscin)	Type I – VIII	AR	256730
Sphingolipidoses (Sphingolipids)	Fabry Disease	X-L	301500
	Farber Disease	AR	228000
	Gaucher Disease	AR	231000
	GM1 Gangliosidoses	AR	230500
	GM2 Gangliosidoses	AR	272800
	Metachromatic leukodystrophy	AR	250100
	Niemann-Pick A, B, C	AR	257200
	Krabbe Disease	AR	245200
Unclassified LSD	Cystinosis	AR	219750
	Cholesteryl ester storage disease	AR	278000
	Free sialic acid deposition disorders	AR	269920
	Multiple sulphatase deficiency	AR	272200
	Pycnodysostosis	AR	265800

*AR: Autosomal recessive. X-LD: X-linked dominant. X-LR: X-linked recessive. *Recently described in Turkish and Yakut (Russian) patients (See [13]). Note that MPS V (Sheie syndrome) is not included in the table since it was later recognized as an attenuated form of MPS I. Similarly, MPS VIII is not included since after being proposed, it was recognized as a laboratory pitfall and the proposal was withdrawn [14].*

LSDs can be classified into 7 groups according to the accumulated general substrate (Table 1).

The diagnosis of a LSDs is based on clinical evaluation of the patient and laboratory tests such as the evaluation of the enzymatic activity [2], or the primary stored substrate (e.g., glycosaminoglycans, oligosaccharides, sphingolipids, and glycogen, among others) [3, 4]. Molecular diagnosis is still difficult considering the lack of complete understanding of the clinical implications of novel variants [5-7]. Nevertheless, molecular diagnosis should be considered during the diagnosis of some LSDs, such as neuronal ceroid lipofuscinosis [8]. Moreover, lyso-Gb3 for Fabry disease (FD) or macrophage inflammatory protein 1-alpha (MIP-1α) in Gaucher disease (GD) have been suggested as novel biomarkers of these LSDs [5]. Chitotriosidase has also been proposed as a biomarker for some LSDs such as GD [9], cystinosis [10], GM1 gangliosidoses [11], and Niemann-Pick type C [12]. Particularly in GD, some hematological alterations on macrophages are frequently found in both smear blood and biologic fluids (i.e., Bronchoalveolar lavage-BAL), which may contribute to the diagnosis. Nevertheless, leukocyte abnormalities also can be found in other LSDs. In this review, we described the major hematological alterations in LSDs found in 56 case reports published between 2010 and 2020. Reference values of all hematological findings were adjusted to the guidelines on standard operating procedures for hematology of the World Health Organization (WHO).

2. LSD AND GENERAL DIAGNOSIS

Lysosomes were first described in the 1950s by Christian De Duve, who recognized the role of this organelle in the degradation and recycling of intracellular and extracellular macromolecules [15]. During the last decade, they have been recognized other lysosomal functions due to the fundamental role as a metabolic hub, influencing and sensing diverse nutrient processes, secretion, gene regulation, plasmatic membrane repair, ionic homeostasis, immune response, and cholesterol transport, among others [16]. The lysosomal biogenesis is triggered by the transcription factor EB (TFEB), which is a master regulator of the lysosomal biogenesis and influences the interaction of the lysosome with other organelles and the cellular homeostasis [16].

LSDs are a group of about 70 monogenic metabolic disorders caused by the deficiency of a specific protein (i.e., enzyme, transporter, or cofactor) involved in the lysosomal function. This deficiency leads to the lysosomal accumulation of partially degraded substrates that generates and spectrum of clinical manifestations depending of the stored substrate and the affected tissues [17]. LSDs have an overall prevalence between 1 in 4,000 and 7,000 live births. Nevertheless, the exact prevalence of LSDs is difficult to calculate due to their wide heterogeneity, which sometimes leads to misdiagnosis [5]. An important group of LSDs are associated with central nervous system (CNS) impairment, while others are mainly characterized by their effect on peripheral organs. The reader is referred to recent review for more details about LSDs [18].

3. DIAGNOSIS

Given the enormous heterogeneity found in the LSDs, the diagnosis is always a challenge, and it requires a common effort between clinicians, biochemists, and medical laboratory scientists to achieve an adequate and timely diagnosis. In this sense, clinical suspicion should be accompanied by accurate routine and specialized laboratories.

3.1 Clinical approach

LSDs clinical manifestation mainly depends on the stored substrate and the affected tissues.

Usually, the storage substrate occurs in the organs where the substrate is synthesized (i.e., liver, spleen, bone, brain, muscle). In this sense, the accumulation of the substrate will generate a disruption of the cellular homeostasis, inducing cellular damage, mitochondrial stress, apoptosis, dysregulation of redox processes, and disruption of the lysosome in/out transport [19-21]. Phenotypic features of patients with LSDs vary depending of the stored substrate and may include coarse face, short stature, skeletal abnormalities, hepatosplenomegaly, cardiac and lung disease, eye and ear impairment, and central nervous system involvement [5, 18].

3.2 Laboratory approach

As previously noted, the diagnostic confirmation of a LSD requires a specialized laboratory including the identification of biomarkers (e.g., stored substrate quantitation), determination of enzymatic activities, and molecular diagnosis. In these scenarios, important advances have been reached. For instance, the measure of GAGs by several qualitative and quantitative methods such as alcian blue, toluidine blue, paper, and thin-layer chromatography, high-pressure chromatography, gas chromatography, mass spectrometry is used in MPS diagnosis and follow up [22]. Collagen type II, β-galactosidase, nidogen-1, and fatty acid-binding protein are also used in MPS [23]. For FD, it has been proposed the globotriaosylsphingosine determination, whereas the measure of chitotriosidase is used for GD. Other biomarkers for GD include glucosylsphingosine, macrophages protein 1-alpha and 1-beta, cathepsin K and osteopontin [23]. For sphingolipidoses such as Krabbe, the psychosine has been described as a biomarker, which may determine the progression of the disease [23], whereas the cholestane-3β, 5α, 6β-triol (C-triol), and 7-ketoholesterol seem to be a sensible biomarker in Niemann-Pick type C (NPC) [24, 25]. In glycogenoses, as in Pompe disease, the most known biomarker is tetrasaccharide glucose (Glc4), which correlates with therapy response. Other two biomarkers, myostatin and insulin-growth factor I (IGF-I), can also be used in Pompe disease [23, 26]. These biomarkers could be useful in pseudo-deficiencies, as reported for MPS I, MPS VII, and GD [27, 28], for which the traditional biochemical tests not always lead to a specific diagnosis. The enzyme activity determination is the gold standard for the diagnosis of LSDs produced by the impairment of an enzyme. This activity can be assayed in a wide range of biological samples such as plasma, serum, leukocytes, cultured fibroblasts, chorionic villi, amniotic fluid, cultured amniocytes, and dried blood spots [29]. Finally, the molecular diagnosis can helpful to establish the diagnosis and genetic counseling interventions [3], and is the gold standard for other conditions as neuronal ceroid lipofuscinosis [8]. Nevertheless, biomarker-, enzyme activity-, or molecular-based diagnosis of LSDs requires highly specialized facilities and trained personnel. In contrast, the analysis of the hematological abnormalities, which can help in the diagnosis of some LSDs, can be performed in low and high-complexity laboratories. In this sense, in the next sections, we will review the complexity of the hematopoietic system and the major alterations reported in some LSDs.

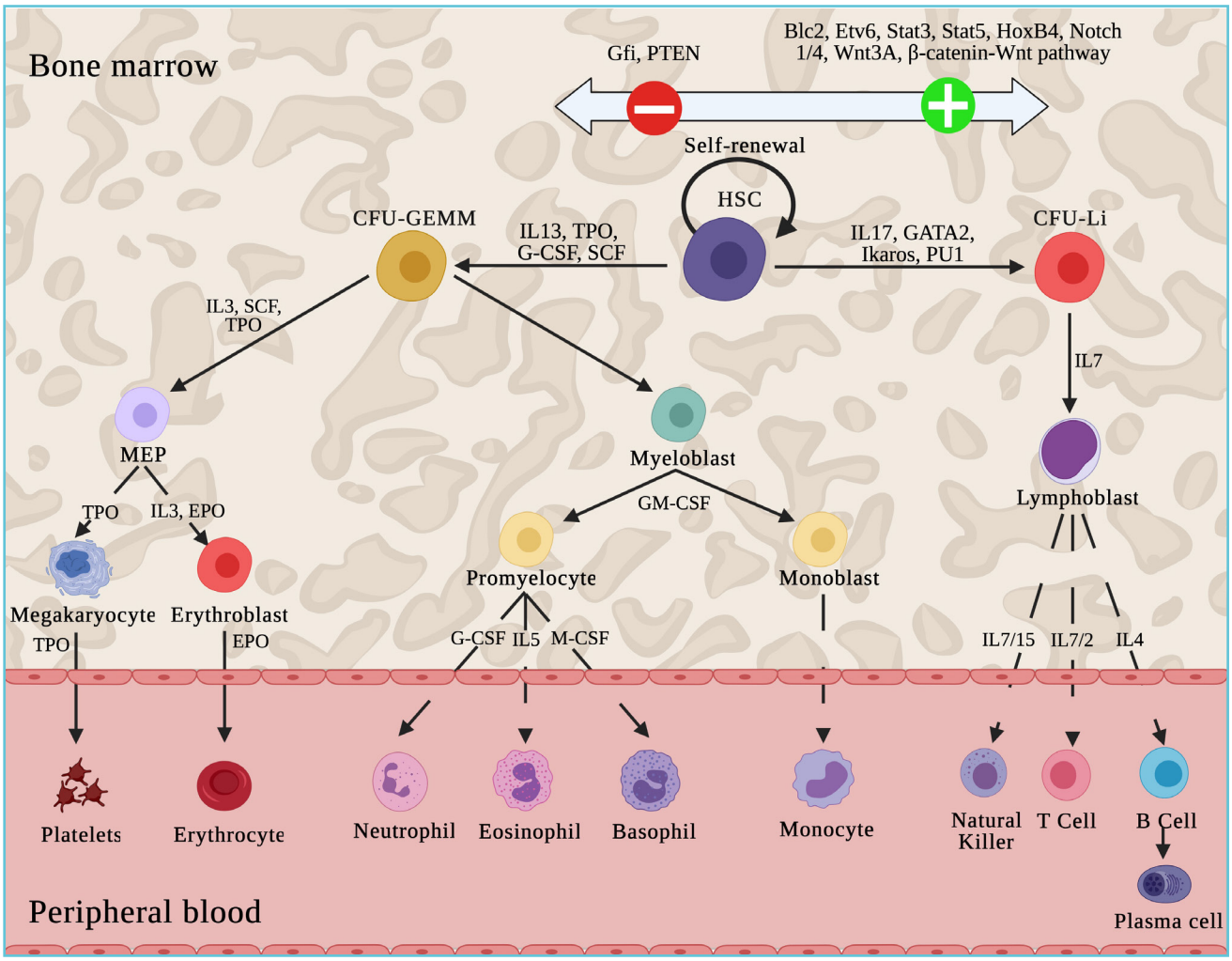
4. OVERVIEW OF THE HEMATOPOIETIC SYSTEM

Hematopoietic stem cells (HSC) are considered the foundation of the adult hematopoietic system and have a crucial role in the long-term maintenance and production of all mature blood cell lineages. Pioneering studies by Till and McCulloch, 1960 and Becker *et al.*, 1963; using transplantation experiments demonstrated the clonality of the adult hematopoietic system

and indicated that the majority of blood cells originate from very few/unique multipotent HSCs capable of proliferation, differentiation, and self-renewal [30]. These HSC-independent hematopoietic cells originate in the embryo and persist in the adult hematopoietic system [31, 32]. Whereas thrombopoietin, erythropoietin, colony-stimulating factor, and some interleukins promote the HSCs proliferation; several cytokines promote the HSC differentiation into multipotential colony-forming progenitor units (CFU) including: CFU-GEMM (Granulocytes, Erythrocytes, Monocytes, Megakaryocytes) for the myeloid lineage and CFU-Li (T, B or NK cells) for the lymphoid lineage (Figure 1) [33, 34].

Since mature blood cells derived from HSC contain all the eukaryotic organelles, including lysosome, substrate accumulation observed in LSDs

Figure 1 **Hematopoietic landscape. HSCs are cells with unique characteristics of self-renewal, pluripotency, and differentiation**



Several pathways have been described like positive (+) and negative (-) modulators of self-renewal [35, 36]. Upon intrinsic and extrinsic signals (cytokines, growth factors, and transcription factors), HSCs can give origin to GFU-GEMM and CFU-Li to myeloid and lymphoid precursors, respectively [37]. In contrast with the BM, where the blood cells precursors are present, in the bloodstream only mature cells are found which are virtually distributed around the body. All the blood cells, except the erythrocyte and platelets, maintain their organelles including the lysosomes. This figure was created using BioRender.com.

can affect not only the hematopoietic precursors but also the most differentiated cells as red blood cells, leukocytes, and thrombocytes or even platelets [38, 39]. In Table 2, we summarize the normal values for the blood count and in the next sections, we describe the normal findings of the blood cells as well as their alteration in the LDS context.

Table 2 Reference values of hemogram

Lineage	Parameter	Units	0-2 years	2-5 years	Men	Women
Red cell lineage	Red blood cells	Cells/µL	4.5±0.8	4.6±0.7	$5x10^6$	$4.5x10^6$
	Hemoglobin	g/L	120 ± 15	125 ± 10	140 - 175	123 - 153
	Hematocrit	%	36 ± 3	37 ± 3	42 - 50	36 - 45
	MCV	fL	78±8	81±6	87±7	
	MHC	pg	27±4	27±3	29±2	
	RDW	%	13.6 ± 2	12.8 ± 1.2	13 ± 1.5	
	Reticulocytes	%	1.0 ± 0.8		<2	
Leukocytes	Leukocyte count	Cells/µL	$6x10^3$ - $7x10^3$	$5.5x10^3$ – $15.5x10^3$	$4x10^3$ - $11x10^3$	
	Lymphocytes	Cells/µL	$3x10^3$ – $9.5x10^3$	$2x10^3$ – $8x10^3$	$1x10^3$ - $4x10^3$	
		%	44 – 74	35 – 65	20 – 40	
	Monocytes	Cells/µL	$0.5x10^3$		$0,15x10^3$ - $0,9x10^3$	
		%	5		2-8	
	Neutrophils	Cells/µL	$1,5x10^3$ - $8,5x10^3$		$2,5x10^3$ - $8,0x10^3$	
		%	15 - 45	25 - 57	55 - 65	
	Eosinophils	Cells/µL	$0,3x10^3$		$0,05x10^3$ - $0,5x10^3$	
		%	3		1 - 3	
	Basophils	Cells/µL	$0,1x10^3$		$0,025x10^3$ - $0,1x10^3$	
		%	0 - 1			
Platelets	Platelets	Cells/µL	$200x10^3$ - $400x10^3$		$150x10^3$ - $450x10^3$	

Values have been adjusted from WHO guidelines [40].

5. HEMATOLOGICAL ABNORMALITIES IN LSDS

We found a total of 51 articles that included 56 LSDs cases in which hematological parameters were evaluated. The reported cases included 26 sphingolipidosis, 13 MPS, 3 ML, 3 neuronal ceroid lipofuscinosis (NCL), 2 glycogenosis, 2 glycoproteinosis, 1 cystinosis, 1 cholesteryl ester storage disease (CESD), and 1 unclassified LSDs. Male patients were more frequently reported than females (57.2% male vs 42.8% female). The age for male patients was between 2 weeks and 68 years; whereas females ranged from newborn to 69 years. We focused on the hematological parameters before the establishment of any clinical intervention to identify baseline values and morphological changes in blood cells that could be associated with the disease. All data is summarized in Table 3.

5.1 Red cell lineage

Anemia was reported in 30.3% of the cases (17/56), with GD having the highest number of reports (64.7%), followed by cystinosis, CESD, NPC, ML IV, MPS I, and aspartylglucosaminuria, with 5.9% each one. Based on the MCV, 3 cases of microcytic anemia (MCV <80fL) were identified, all of them corresponding to GD. Although mild anemia was the main finding, one case reported a severe microcytic hypochromic anemia (Hb: 50 g/L; MCV: 66 fL, MCH: 19.4 pg) in a 1-year-old man diagnosed with GD type 1 with slight anisocytosis and poikilocytosis [41]. Interestingly, a 69 years-old Japanese female with GD type 1 and gastric cancer had a reduction in the erythrocytes count ($3.98x10^6$/µL) and hematocrit levels (34,6%), with marked reticulocytosis (20%) [42]. In this patient, a novel mutation c.587A>G (p.K157R) in the *GBA* gene was reported; however, the impact of this mutation on the β-glucocerebrosidase was not evaluated.

5.2 Leukocytes

As previously described, LSDs are a group of metabolic disorders characterized by substrate accumulation into the lysosome [1]. In this sense, it could be expected that the major hematological findings are related to the leukocyte morphology due to the presence of organelles in these cells [43]. The reader is referred to some of the reviewed publications for representative images of leukocytes alterations [44-48].

5.2.1 Granulocytes

Basophil abnormalities were not reported on any of the revised cases. On the other hand, eosinophils with enlarged and sparse granules in the blood smear were reported in three cases of GM1 gangliosidoses [44, 49, 50]. Although these alterations have been recognized since early reports as frequent alterations of GM1 gangliosidoses [51], they are not routinely investigated as part of suspicion of GM1 gangliosidoses unless the absolute leukocyte count is altered. Eosinophils with deep pink-blue granules were reported in a MPS type VI female patient, as well as abnormal lobulation on neutrophils [52]. These findings were observed after an alteration in the automated analysis, which led to a suspicion that was later confirmed as pseudobasophilia. On the other hand, the Alder-Reilly anomaly was identified on neutrophils from MPS I, IV, and VI patients [45, 52-55], representing a potential finding for this LSD group. Although we did not find the Alder-Reilly anomaly in the reports for other LSDs, it is not possible to discard its presence in entities different than MPS. In addition, Alder-Reilly inclusions must not be confused with toxic granulations that are restricted to neutrophils in a transitory form during an inflammatory process [43].

5.2.2 Monocytes

Despite the crucial role of monocytic cells, morphology alterations were not frequently

reported in the peripheral blood of LSDs patients. However, some dense blue/purple-black granules have been occasionally reported in monocytes from MPS patients [54, 55], which are metachromatic after toluidine staining [46]. Nevertheless, the most common findings on monocytes seem to be restricted to the phagocytic activity of the macrophages from BM, liver, and spleen. These changes will be discussed later.

5.2.3 Lymphocytes

Morphologic alterations in lymphocytes were reported in almost all the LSDs cases reviewed. Vacuolated lymphocytes were reported in GM1 gangliosidoses [49, 56], ML [57], and MPS III [58]. This vacuolization was accompanied by Alder-Reilly inclusions in MPS IIIA [47], MPS IIIB [59], MPS IV [54] and MPS VII [59]. In ML type II (I-cell disease) vacuole-like inclusions are frequently observed on lymphocytes, in which accumulation of HLA class II molecules have been observed, suggesting a role of N-acetylglucosamine-1-phosphotransferase in the immune system [60]. In this sense, the study of the chemical nature of the inclusions may increase our knowledge about the physiopathology of the disease and lead to the identification of novel therapeutic targets. On the other hand, atypical and reactive states of lymphocytes were reported in one case of GD [61] and aspartylglucosaminuria [62], respectively. Also, electronic microscopy of peripheral blood samples allowed the identification of cytoplasmatic vacuoles containing electrodense periodic structures in lymphocytes from NCL patients [63-65].

In Pompe disease, it has been reported that vacuolation of lymphocytes [66] is a consequence of the lysosomal glycogen accumulation [67]. Electronic microscopy of peripheral blood cells, allowed the identification of inclusions in samples from Pompe disease patients [68]. Despite vacuolization is being suggestive of lysosomal storage, the use of a specific glycogen staining (i.e., periodic acid-Schiff, PAS), allows a more accurate diagnostic impression. This was validated by Hagemans *et al*., through the analysis of PAS-stained blood films obtained from 65 patients with classical infantile and adult forms of Pompe disease, with a sensitivity and specificity of 100% and 98%, respectively [69].

5.3 Platelets

We found platelet counts in 25 out of the 56 case reports reviewed. In 60% of the cases (15/25), it was reported a marked thrombocytopenia (< 95 $x10^3$cells/µL), without abnormalities in the morphology. Although most of the reports are from GD patients (7/15) [48, 70, 71], it was also reported in Niemann-Pick type B [72] and C [73]; MPS II [74], MPS VII [75], aspartylglucosaminuria [62], and cystinosis [76]. One case of thrombocytosis (595 $x10^3$cells/µL) was reported in a 5 months-old female Pompe disease patient [66], whereas in one case the use of electronic microscopy allowed the visualization of granular inclusions on platelets from a 59 years-old Pompe disease patient [68].

5.4 Leukocyte abnormalities on non-peripheral blood cells

The major goal of this review article was to describe the more common hematological findings in LSDs with a particular focus on the blood smear due to its routine use in medical laboratories. Nevertheless, interesting changes of leukocytes, particularly in monocyte-derived lineage, have been registered in several biological samples which are summarized in Table 4. For instance, for GD it has been described the presence of Gaucher cells in BM [80, 86]. Gaucher cells are very large cells, usually macrophages, with a diameter between 20 and 80 µm with a small and eccentrically placed nucleus as well as a cytoplasm with wrinkles or striations [87].

Table 3 Summary of hematological abnormalities in LSDs patients

LSD	Red cell lineage	White line				Platelets	Ref.
		Eosino-phils	Neutro-phils	Mono-cytes	Lympho-cytes		
Gaucher	Anemia				Atypical	Thrombocytopenia	[41, 48, 61, 70, 71, 77-80]
Niemann-Pick	Anemia					Thrombocytopenia	[72, 73, 81, 82]
GM1 Gangliosidoses		Enlarged and sparse granules			Vacuolated		[44, 49, 50, 56]
Pompe					PAS-positive vacuoles	Thrombocytosis	[66, 68, 69]
Cystinosis	Anemia					Thrombocytopenia	[76]
CESD	Anemia						[83]
MPS I			AR				[45]
MPS II			AR			Thrombocytopenia	[53, 74]
MPS III A-B					Vacuoles with AR		[47, 58, 59]
MPS IV			AR	Blue/ purple-black granules	AR		[54]
MPS VI		Deep pink-blue granules	Abnormal lobulation and AR	Red-violet granules			[46, 52, 55]
MPS VII					Vacuoles with AR	Thrombocytopenia	[75, 84]
ML II					Vacuolated		[57, 60]
ML IV	Anemia						[85]
Asp.	Anemia				Reactive	Thrombocytopenia	[62]

MPS: Mucopolysaccharidosis. ML: Mucolipidosis. CESD: Cholesteryl ester storage disease. AR: Alder-Reilly anomaly. Asp: Aspartylglucosaminuria.

Table 4 Leukocyte abnormalities in non-peripheral blood samples from LSD patients

Sample	Technique	Leukocyte lineage	Findings	LSD	Reference
BM	GS	Myeloid	Histiocytes containing refractile crystal in the cytoplasm	Cystinosis	[76]
	HES	Myeloid	*Gaucher cells	Gaucher disease	[41, 48, 77, 79, 86, 92, 93]
	EM	Myeloid	*Gaucher cells	Gaucher disease	[80]
	APS	Myeloid	Histiocytes strongly positive	Gaucher disease	[42]
	GS	Myeloid	Foamy macrophages	Niemann Pick disease	[81, 88]
	TBS	Myeloid	Macrophages with metachromatic granules	MPS III	[58]
	WRS/GS	Lymphoid	**Azurophilic inclusions type dots or commas in plasma cells	MPS IIIA	[47]
Spleen	HES	Myeloid	*Gaucher cells	Gaucher disease	[70]
BAL	PPS	Myeloid	Foamy macrophages	Niemann Pick disease	[89]
Liver biopsy	NA	Myeloid	Foamy macrophages	Niemann Pick disease	[90]
CSF	WRS/GS	Myeloid	Intracytoplasmic granules in macrophages with haloes	MPS I	[91]

*BM: Bone marrow. BAL: Bronchoalveolar lavage. CFS: Cerebrospinal fluid. MPS: Mucopolysaccharidosis. NA: Not available. GS: Giemsa staining. HES: hematoxylin and eosin staining. EM: Electron microscopy. APS: Acid phosphatase stain. TBS: Toluidine blue staining. WRS: Wright staining. PPS: Papanicolaou stain. *Despite these cells could be suggestive of Gaucher disease, the presence of pseudo-Gaucher cells in bone marrow can address an erroneous diagnosis impression when routine hematoxylin-eosin staining is performed. To identify true Gaucher cells, iron staining is recommended and typically the presence of diffuse iron staining should be observed in Gaucher cells which is absent on pseudo-Gaucher cells [94]. **These findings are compatible with MPS I, II, and III when are present on peripheral lymphocytes; however, if these inclusions are found in plasma cells on bone marrow the major clinical suspicion is MPS III.*

Likewise, in Niemann Pick disease type B and C foamy macrophages have been observed in BM, bronchoalveolar lavage, and liver biopsy [88-90]. Histiocytes with refractile crystal in BM [76], and macrophages with intracytoplasmic granules in cerebrospinal fluid [91], were found in cystinosis and MPS I, respectively. Finally, azurophilic inclusions in plasma cells were found in BM from MPS IIIA patients [47].

6. CONCLUSIONS AND PERSPECTIVES

In LSDs, the accumulation of undegraded macromolecules into the lysosome triggers complex pathogenetic cascades leading to the clinical manifestations of these diseases. Impaired metabolism of lysosomal substrates may affect the autophagic-lysosomal system, the ubiquitin-proteosome system, the lysosome membrane permeability, and promotes inclusion body formation, dysregulation of the signaling pathway, inflammation, calcium homeostasis abnormalities, mitochondrial dysfunction, and oxidative stress [18].

These cellular changes may affect several tissues leading to different clinical manifestations, including hematological alterations. Although these hematological alterations may not be common findings in LSDs, they may contribute to the diagnosis of the disease, especially considering that the tests to evaluate these hematological abnormalities are available in most clinical laboratories. Nevertheless, health care professionals need to be aware of hematological alterations and correlate them with patient symptoms and other clinical laboratory results. In addition, it is necessary to extend the study of hematological alterations to other LSDs to explore the potential of these abnormalities as a biomarker for diagnosis or treatment follow-up.

❖❖❖❖❖

Research funding

A.F.L. received a doctoral scholarship from Pontificia Universidad Javeriana. C.J.A.D was supported by the Ministry of Science, Technology, and Innovation, Colombia (Grant ID 120380763212 – PPTA # 8352), Pontificia Universidad Javeriana (PPTA # 8275, 20289, and 20300), and the National MPS Society (PPTA # 9507).

Author contributions

A.F.L., W.G.N., E.C., H.P., wrote the original draft. A.F.L., C.J.A.D., reviewed and edited the manuscript. All authors contributed to the literature analysis. All authors have read and approved the final manuscript.

Competing interests

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the review.

❖❖❖❖❖

REFERENCES

1. Leal, A.F., et al., Lysosomal storage diseases: current therapies and future alternatives. J Mol Med (Berl), 2020: p. 931-946.

2. Mokhtariye, A., et al., Diagnostic methods for Lysosomal Storage Disease. Rep Biochem Mol Biol, 2019. 7(2): p. 119-128.

3. Filocamo, M. and A. Morrone, Lysosomal storage disorders: molecular basis and laboratory testing. Hum Genomics, 2011. 5(3): p. 156-69.

4. Winchester, B., Lysosomal diseases: diagnostic update. J Inherit Metab Dis, 2014. 37(4): p. 599-608.

5. Poswar, F.O., et al., Lysosomal diseases: Overview on current diagnosis and treatment. Genet Mol Biol, 2019. 42(1 suppl 1): p. 165-177.

6. Bekri, S. Laboratory diagnosis of lysosomal storage diseases. 2006 [cited 2020 October/13]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK11594/.

7. Giugliani, R., et al., Current molecular genetics strategies for the diagnosis of lysosomal storage disorders. Expert Rev Mol Diagn, 2016. 16(1): p. 113-23.

8. Gheldof, A., et al., Clinical implementation of gene panel testing for lysosomal storage diseases. Mol Genet Genomic Med, 2019. 7(2): p. e00527.

9. Sperb-Ludwig, F., et al., Chitotriosidase on treatment-naïve patients with Gaucher disease: A genotype vs phenotype study. Clin Chim Acta, 2019. 492: p. 1-6.

10. Veys, K.R.P., et al., Chitotriosidase as a Novel Biomarker for Therapeutic Monitoring of Nephropathic Cystinosis. J Am Soc Nephrol, 2020. 31(5): p. 1092-1106.

11. Arash-Kaps, L., et al., The Clinical and Molecular Spectrum of GM1 Gangliosidosis. J Pediatr, 2019. 215: p. 152-157.e3.

12. Sitarska, D. and A. Ługowska, Laboratory diagnosis of the Niemann-Pick type C disease: an inherited neurodegenerative disorder of cholesterol metabolism. Metab Brain Dis, 2019. 34(5): p. 1253-1260.

13. Vasilev, F., A. Sukhomyasova, and T. Otomo, Mucopolysaccharidosis-Plus Syndrome. Int J Mol Sci, 2020. 21(2).

14. Giugliani, R., et al., Overview of Mucopolysaccharidoses: Diagnosis, Natural History, Clinical pictures, in Mucopolysaccharidoses Update (2 Volume Set), S. Tomatsu, et al., Editors. 2018, Nova Science Publishers, Inc.: Hauppauge, NY. p. 17-19.

15. DE DUVE, C., et al., Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem J, 1955. 60(4): p. 604-17.

16. Bajaj, L., et al., Lysosome biogenesis in health and disease. J Neurochem, 2019. 148(5): p. 573-589.

17. Sun, A., Lysosomal storage disease overview. Annals of translational medicine, 2018. 6(24): p. 476.

18. Parenti, G., D.L. Medina, and A. Ballabio, The rapidly evolving view of lysosomal storage diseases. EMBO Mol Med, 2021. 13(2): p. e12836.

19. Myerowitz, R., R. Puertollano, and N. Raben, Impaired autophagy: The collateral damage of lysosomal storage disorders. EBioMedicine, 2021. 63: p. 103166.

20. Stepien, K.M., et al., Mechanisms of Mitochondrial Dysfunction in Lysosomal Storage Disorders: A Review. J Clin Med, 2020. 9(8).

21. Fecarotta, S., et al., Pathogenesis of Mucopolysaccharidoses, an Update. Int J Mol Sci, 2020. 21(7).

22. Kubaski, F., et al., Glycosaminoglycans detection methods: Applications of mass spectrometry. Mol Genet Metab, 2017. 120(1-2): p. 67-77.

23. Bobillo Lobato, J., M. Jiménez Hidalgo, and L.M. Jiménez Jiménez, Biomarkers in Lysosomal Storage Diseases. Diseases, 2016. 4(4).

24. Hammerschmidt, T.G., et al., Molecular and biochemical biomarkers for diagnosis and therapy monitorization of Niemann-Pick type C patients. Int J Dev Neurosci, 2018. 66: p. 18-23.

25. Boenzi, S., et al., Evaluation of plasma cholestane-3β,5α,6β-triol and 7-ketocholesterol in inherited disorders related to cholesterol metabolism. J Lipid Res, 2016. 57(3): p. 361-7.

26. Chien, Y.H., et al., Myostatin and insulin-like growth factor I: potential therapeutic biomarkers for pompe disease. PLoS One, 2013. 8(8): p. e71900.

27. Kubaski, F., et al., Neonatal Screening for MPS Disorders in Latin America: A Survey of Pilot Initiatives. Int J Neonatal Screen, 2020. 6(4).

28. Mashima, R. and T. Okuyama, Enzyme activities of α-glucosidase in Japanese neonates with pseudodeficiency alleles. Mol Genet Metab Rep, 2017. 12: p. 110-114.

29. McPherson, R.A. and M.B. Pincus, Diagnósticos clínicos e tratamento por métodos laboratoriais de Henry. 2012.

30. Sharkis, S.J., Canadian stem cell scientists take the prize. Cell, 2005. 122(6): p. 817-9.

31. Medvinsky, A., S. Rybtsov, and S. Taoudi, Embryonic origin of the adult hematopoietic system: advances and questions. Development, 2011. 138(6): p. 1017-31.

32. Dzierzak, E. and A. Bigas, Blood Development: Hematopoietic Stem Cell Dependence and Independence. Cell Stem Cell, 2018. 22(5): p. 639-651.

33. Cheung, A.M., et al., Stem cell model of hematopoiesis. Curr Stem Cell Res Ther, 2006. 1(3): p. 305-15.

34. Höfer, T. and H.R. Rodewald, Differentiation-based model of hematopoietic stem cell functions and lineage pathways. Blood, 2018. 132(11): p. 1106-1113.

35. Duan, Z. and M. Horwitz, Gfi-1 takes center stage in hematopoietic stem cells. Trends Mol Med, 2005. 11(2): p. 49-52.

36. Akala, O.O. and M.F. Clarke, Hematopoietic stem cell self-renewal. Curr Opin Genet Dev, 2006. 16(5): p. 496-501.

37. Scharf, P., et al., Cellular and Molecular Mechanisms of Environmental Pollutants on Hematopoiesis. Int J Mol Sci, 2020. 21(19).

38. Benjamin, R.J. and L.S. McLaughlin, Plasma components: properties, differences, and uses. Transfusion, 2012. 52 Suppl 1: p. 9S-19S.

39. Mathew J, Sankar P, and V. M., Physiology, Blood Plasma. StatPearls, 2020: p. 1-7.

40. WHO. World Health Organization. Regional Office for South-East Asia. Guidelines on standard operating procedures for haematology. 2000.

41. Kim, E.A., et al., Neuronopathic Gaucher disease presenting with microcytic hypochromic anemia. Int J Hematol, 2019. 109(3): p. 361-365.

42. Hosoba, S., et al., A novel mutation causing type 1 Gaucher disease found in a Japanese patient with gastric cancer: A case report. Medicine (Baltimore), 2018. 97(27): p. e11361.

43. Marionneaux, S., Nonmalignant leukocyte disorders. Rodak's Hematology, 2020.

44. Chevalier, C. and G. Detry Vacuolated lymphocytes and abnormal eosinophils in GM1 gangliosidosis, type 1. British journal of haematology, 2012. 156: p. 293-293.

45. Bhuyan, P., et al., Alder-Reilly Anomaly in Hurler's Syndrome in a Neonate: A Rare Case Report. Indian J Hematol Blood Transfus, 2013. 29(3): p. 184-6.

46. Krishnagiri, C., et al., Abnormal granulation of blood granulocytes in mucopolysaccharidosis VI-a case report. Ann Diagn Pathol, 2013. 17(1): p. 137-9.

47. Nava-Aguilera, M.L., et al., Azurophilic inclusions in lymphocytes and plasma cells: a case of Sanfilippo disease. Br J Haematol, 2014. 164(5): p. 618.

48. Du, X., et al., Three mutations of adult type 1 Gaucher disease found in a Chinese patient: A case report. Medicine (Baltimore), 2018. 97(47): p. e13161.

49. Lynch, D.T. and D.R. Czuchlewski. Peripheral blood findings in GM1 gangliosidosis. 2016 [cited 2020 November, 15th].

50. Salama, R. and J. Zhou, Vacuolated lymphocytes signifying a metabolic disorder in an infant with developmental delay. Clin Case Rep, 2016. 4(1): p. 99-100.

51. Gitzelmann, R., et al., Anomalous eosinophil granulocytes in blood and bone marrow: a diagnostic marker for infantile GM1-gangliosidosis? Eur J Pediatr, 1985. 144(1): p. 82-4.

52. Piva, E., et al., The role of automated analyzers in detecting abnormal granulation of leucocytes in lysosomal storage diseases: Maroteaux-Lamy disease. Am J Hematol, 2013. 88(6): p. 527.

53. AlMozain, N. and N.A. Bakshi, A case of hunter syndrome and Alder-Reilly anomaly. Journal of Applied Hematology, 2017. 8: p. 33-35.

54. Jain, R., et al., Mucopolysaccharidosis: A case report highlighting hematological aspects of the disease. J Lab Physicians, 2019. 11(1): p. 97-99.

55. Teixeira, C., J. Barbot, and M.I. Freitas, From blood film to the diagnosis of rare hereditary disorders. Br J Haematol, 2015. 168(3): p. 315.

56. Vasei, M., M. Abolhasani, and M. Safavi, Vacuolated Lymphocytes as a Clue for Diagnosis of Lysosomal Storage Disease like GM1 Gangliosidosis. Indian J Hematol Blood Transfus, 2018. 34(4): p. 749-750.

57. Bounds, R.L., et al., Left Main Coronary Artery Atresia in an Infant With Inclusion-Cell Disease. World J Pediatr Congenit Heart Surg, 2018. 9(2): p. 246-250.

58. Ireland, R.M., Morphology in mucopolysaccharidosis type III: specific diagnostic features. Blood, 2016. 127(5): p. 662.

59. Do, L. and L. Pasalic, Lymphocytes in Sanfilippo syndrome display characteristic Alder-Reilly anomaly. Blood, 2019. 134(14): p. 1194.

60. Yokoi, A., et al., B-cell-specific accumulation of inclusion bodies loaded with HLA class II molecules in patients with mucolipidosis II (I-cell disease). Pediatr Res, 2019. 86(1): p. 85-91.

61. Rizk, T.M., R.O. Ariganjoye, and G.I. Alsaeed, Gaucher disease. Unusual presentation and mini-review. Neurosciences (Riyadh), 2015. 20(3): p. 271-6.

62. Liu, Y., et al., [A family with two children diagnosed with aspartylglucosaminuria-case report and literature review]. Zhonghua Er Ke Za Zhi, 2014. 52(6): p. 455-9.

63. Lau, N.K.C., et al., First case of genetically confirmed CLN3 disease in Chinese with cDNA sequencing revealing pathogenicity of a novel splice site variant. Clin Chim Acta, 2018. 486: p. 151-155.

64. Canafoglia, L., et al., Recurrent generalized seizures, visual loss, and palinopsia as phenotypic features of neuronal ceroid lipofuscinosis due to progranulin gene mutation. Epilepsia, 2014. 55(6): p. e56-9.

65. Guerreiro, R., et al., CLN6 disease caused by the same mutation originating in Pakistan has varying pathology. Eur J Paediatr Neurol, 2013. 17(6): p. 657-60.

66. Bajel, A., et al., Lymphocyte vacuolation: clue to inherited metabolic disease. Pathology, 2010. 42: p. 699-700.

67. Morales, J. and A. Anilkumar Glycogen Storage Disease Type II (Pompe Disease). 2020.

68. Hossain, M.A., et al., A Case of Adult-onset Pompe Disease with Cerebral Stroke and Left Ventricular Hypertrophy. J Stroke Cerebrovasc Dis, 2018. 27(11): p. 3046-3052.

69. Hagemans, M.L., et al., PAS-positive lymphocyte vacuoles can be used as diagnostic screening test for Pompe disease. J Inherit Metab Dis, 2010. 33(2): p. 133-9.

70. Yang, M., Fever, pulmonary interstitial fibrosis, and hepatomegaly in a 15-year-old boy with Gaucher disease: a case report. J Med Case Rep, 2018. 12(1): p. 306.

71. Mazher, W., et al., Improvement In Symptoms Of Gaucher's Disease By Enzyme Replacement Therapy. J Ayub Med Coll Abbottabad, 2018. 30(3): p. 479-481.

72. Grasko, Y., et al., A novel missense SMPD1 gene mutation, T460P, and clinical findings in a patient with Niemann-Pick disease type B presenting to a lipid disorders clinic. Ann Clin Biochem, 2014. 51(Pt 5): p. 615-8.

73. Kumagai, T., et al., A case of Niemann-Pick disease type C with neonatal liver failure initially diagnosed as neonatal hemochromatosis. Brain Dev, 2019. 41(5): p. 460-464.

74. Uz, B., H. Demiroglu, and O.I. Ozcebe, Hunter syndrome and new onset idiopathic thrombocytopenic purpura in a young patient. Ann Hematol, 2012. 91(2): p. 303-4.

75. Huang, Y.L., et al., [Mucopolysaccharidosis VII: report of a case and review of the literature]. Zhonghua Er Ke Za Zhi, 2011. 49(6): p. 455-8.

76. Lyou, Y., X. Zhao, and C.S. Nangia, Pancytopenia in a patient with cystinosis secondary to myelosuppression from cystine crystal deposition: a case report. J Med Case Rep, 2015. 9: p. 205.

77. Sgherza, N., et al., Who seeks finds. Gaucher's disease: a rare case of thrombocytopenia. Int J Hematol, 2020. 111(3): p. 327-328.

78. Emanuel, A.J., et al., Small Bowel Mucosal Involvement and Mesenteric Mass Formation in a Young Female with Type 3 Gaucher Disease. A Case Report. J Gastrointestin Liver Dis, 2018. 27(4): p. 459-463.

79. Ceravolo, F., et al., Combination therapy in a patient with chronic neuronopathic Gaucher disease: a case report. J Med Case Rep, 2017. 11(1): p. 19.

80. Lo, S.M., et al., Phenotype diversity in type 1 Gaucher disease: discovering the genetic basis of Gaucher disease/hematologic malignancy phenotype by individual genome analysis. Blood, 2012. 119(20): p. 4731-40.

81. Bajaj, S., et al., Rare disease heralded by pulmonary manifestations: Avoiding pitfalls of an "asthma" label. J Postgrad Med, 2017. 63(2): p. 122-127.

82. Bermejo, N., J. Prieto, and M.J. Arcos, Sea-blue histiocytosis in bone marrow of a patient with chronic thrombocytopenia. Acta Haematol, 2015. 133(3): p. 277-8.

83. Canbay, A., et al., Cholesteryl Ester Storage Disease: Fatal Outcome without Causal Therapy in a Female Patient with the Preventable Sequelae of Progressive Liver Disease after Many Years of Mild Symptoms. Am J Case Rep, 2018. 19: p. 577-581.

84. Dubot, P., et al., First Report of a Patient with MPS Type VII, Due to Novel Mutations in GUSB, Who Underwent Enzyme Replacement and Then Hematopoietic Stem Cell Transplantation. Int J Mol Sci, 2019. 20(21).

85. Yamaguchi, N., et al., Novel compound heterozygous MCOLN1 mutations identified in a Japanese girl with severe developmental delay and thin corpus callosum. Brain Dev, 2020. 42(3): p. 298-301.

86. Erdem, N., A. Çizmecioğlu, and İ. Aydoğdu, From Bone Marrow Necrosis to Gaucher Disease; A Long Way to Run. Turk J Haematol, 2015. 32(4): p. 373-4.

87. Gözdaşoğlu, S., Gaucher Disease and Gaucher Cells. Turk J Haematol, 2015. 32(2): p. 187-8.

88. Velarde-Félix, J.S., et al., Pathogenic Compound Heterozygous Mutations in a Mexican Mestizo Patient with Niemann-Pick Disease Type B. Genet Couns, 2016. 27(2): p. 211-7.

89. Chebib, N., F. Thivolet-Bejui, and V. Cottin, Interstitial Lung Disease Associated with Adult Niemann-Pick Disease Type B. Respiration, 2017. 94(2): p. 237-238.

90. Sheth, J., et al., Pulmonary manifestations in Niemann-Pick type C disease with mutations in NPC2 gene: case report and review of literature. BMC Med Genet, 2017. 18(1): p. 5.

91. Lukefahr, A.L. and M. Proytcheva, Alder-Reilly Anomaly in the Cerebrospinal Fluid of a Child With Hurler Syndrome. J Pediatr Hematol Oncol, 2018. 40(1): p. 74-75.

92. Machaczka, M., et al., Recurrent pulmonary aspergillosis and mycobacterial infection in an unsplenectomized patient with type 1 Gaucher disease. Ups J Med Sci, 2014. 119(1): p. 44-9.

93. Liu, L.Y., et al., A Novel Functional Missense Mutation p.T219A in Type 1 Gaucher's Disease. Chin Med J (Engl), 2016. 129(9): p. 1072-7.

94. Gören Şahin, D., et al., Gaucher Cells or Pseudo-Gaucher Cells: That's the Question. Turk J Haematol, 2014. 31(4): p. 428-9.

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

This is a Platinum Open Access Journal distributed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Evaluation of dual marker approach using heart-type fatty acid binding protein and high sensitivity troponin-I as an alternative to serial sampling for diagnosis of acute myocardial infarction

Manish Raj Kulshrestha[1], Apurva Raj[2], Vandana Tiwari[1], Subrat Chandra[2], Bhuwan Chandra Tiwari[3], Ashish Jha[3]

[1] *Department of Biochemistry, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India*
[2] *Department of Pathology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India*
[3] *Department of Cardiology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India*

ARTICLE INFO

Corresponding author:
Dr. Vandana Tiwari
Professor & Head
Department of Biochemistry
Dr. Ram Manohar Lohia Institute
of Medical Sciences
Lucknow, 226010
India
Phone: +919415197228
E-mail: drvandana2166@yahoo.com

Key words:
acute myocardial infarction, heart-type fatty acid binding protein, high sensitive Troponin-I, Dual Marker Approach, Double Sampling Approach

ORCID:
0000-0003-1961-7257

ABSTRACT

Objective

An early rule in (high specificity and high PPV) and early rule out (high sensitivity and high NPV) is essential for diagnosing acute myocardial infarction (AMI) to provide better utilization of resources, cost-effectiveness, and to reduce mortality.

Methods

Consecutive chest pain patients (n=80) with symptoms indicative of coronary artery disease reported to the emergency room within 6 hours after onset of symptoms. An alternate Dual Marker Approach (DMA; both Heart-type Fatty Acid Binding Protein (H-FABP) and High sensitive Troponin-I (hsTnI) at 0 h) was compared

to the Double Sampling approach (DSA; hsTnI at 0 h and 3 h (ESC guidelines)).

Results

If both biomarkers were increased (n=17; 77.5%: 11 STEMI and 6 NSTEMI) above their respective cut-off value (HFABP 6.3 ng/mL and hsTnI 20.24 ng/L) at presentation, AMI ensued (100% PPV). Also, if both the markers were below their respective cut-offs at presentation, AMI was safely ruled out (n=41; with only 1 false negative). However, among the patients with either of these markers above their respective cut-off at presentation (n=22), DSA was required to find remaining AMI cases (n=4). Overall, DMA stands best for rule out (sensitivity 95.5%, NPV 97.6%) while DSA is superior for rule in (98.2% specificity, 95.2% PPV).

Conclusion

With the use of the proposed DMA, 58/80 (72.5%) patients with acute chest pain were reliably ruled in/ruled out for AMI at the presentation itself, while the remaining patients still required serial monitoring (DSA) for confirmation.

❖❖❖❖❖

INTRODUCTION

Early diagnosis and treatment of acute myocardial infarction (AMI) cases during the first hour (golden hour) after symptoms may reduce mortality from 9% to 3% [1]. Similarly, identifying patients without AMI and safely sending them home may result in considerable advantages for both patients and hospitals. ECG changes (ST elevation) and cardiac troponins (cTn), though highly specific, may not be apparent in the initial hours. Previous studies revealed that ECG misinterpretation might result in inappropriate clinical management in about 3-18% of patients[1-3]. Likewise, an inadequate increase in cTn and increasing duration may occur in cases of microinfarction[4].

The continuous improvement of the analytical sensitivity and assay precision in the low measuring range of cardiac troponin (cTn) assays has ultimately led to the development of 'high-sensitivity troponin (hsTnI and hsTnT) assays[5]. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) task force suggested that cTn should be reliably measurable in >80% (preferably >95%) of healthy subjects, and the total imprecision (CV) at the 99th percentile value should be ≤10% [5-7]. High-sensitivity cardiac troponin I (hsTnI) assays measure the same analyte as its predecessor cardiac Troponin I (cTn-I) with limits of detection (LoD) in ranges that were previously undetectable.

This additional sensitivity leads to the detection of cTn release at an earlier time point than the previous generations of cTn assays, especially in patients with a recent onset of chest pain. Most patients with AMI can be reliably identified within 3 h after admission, which indicates that the observation time in the emergency department may be reduced to rule out AMI[8]. However, in patients with 3 h values unchanged, in whom the pre-test likelihood of AMI is high, additional subsequent sampling (e.g., after 2 or 3 h) may still be advisable[5]. However, the sensitivity of the 99th percentile to rule-out AMI is too low for clinical use[9], and diagnosis of AMI cannot be made solely based on troponin I individual test results[5]. Thus, the role of early rule in (high positive predictive value) and early rule out (high sensitivity) algorithms is critical.

Heart-type fatty acid-binding protein (H-FABP), a novel biomarker, is one of the most abundant proteins in cardiomyocytes, comprising 5-15% of the total cytosolic protein pool. It leaks out of myocardial tissue. The concentration increases in the blood within 2 hours and is reported to peak at approximately 4-6 hours and returns to

the normal baseline value in 20 hours[10]. Recent studies have shown that combined with well-established markers such as hsTnI, H-FABP may allow early and accurate rule out compared to the currently available diagnostic tests[11].

The present study aimed at two different algorithms in terms of diagnostic performance and ROC analysis among the acute chest pain patients presenting within 6 hours of onset of symptoms. First, the Double Sampling Approach (DSA), i.e., collecting two samples for hsTnI drawn at presentation (at 0 hour) and after 3 hours. The second, Dual Marker Approach (DMA) estimates hsTnI and HFABP simultaneously at presentation (at 0 hour).

MATERIAL AND METHODS

Patient's selection

This prospective observational study included 80 consecutive cases of chest pain suggestive of coronary origin according to the 2015 American Heart Association guidelines[12] and presenting within 6 hours of the onset of symptoms at the emergency ward of Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow. Patients were enrolled after obtaining written informed consent from the patient's attendant in the format approved by the institutional ethical committee (IEC-31/16). Patients <18 years, eGFR<60 ml/min, unwillingness to provide consent, history of previous AMI, or acute muscle injury/ trauma were excluded from this study, i.e. their samples were not further assessed for HFABP. Comprehensive history taking included the patient's symptoms and time of onset. Past medical history such as diabetes mellitus (DM), hypertension (HTN), and previous ischemic events and general clinical examination, ECG, and laboratory investigations were documented at admission using medical records. However, patients with ST-elevation were analyzed separately.

Diagnostic criteria for acute myocardial infarction (AMI)

The definitive diagnosis was made after a critical review of all the clinical features and relevant information by a panel of two cardiologists. AMI was defined according to the Joint European Society of Cardiology (ESC)/American College of Cardiology (ACC)/American Heart Association (AHA)/World Heart Federation (WHF) Task Force for the Universal Definition of Myocardial Infarction. Presence of at least one element of clinical evidence of acute myocardial ischemia (symptoms of ischemia, ECG findings, coronary angiography, or imaging evidence) and with acute myocardial injury characterized by detection of a rise and/or fall of cTnI values with at least one value above the 99th percentile URL (0.2 ng/ml for Siemens Advia Centaur XP c-TnI assay)[13].

Sample collection

Venous samples (3mL) were taken at presentation ('0' hour) and post-admission (at 3 h) serially for hsTnI estimation (Double Sampling Approach; DSA). After accomplishing routine analysis, including cardiac troponin I (cTnI), the first sample ('0' hour) was stored at -80ºC for H-FABP estimation.

Estimation of hsTnI and H-FABP biomarkers

The first sample (0h) was analyzed for hsTnI and H-FABP both (Dual Marker Approach; DMA), while the second sample at post-admission (3 h) was analyzed for hsTnI only. According to the manufacturer's protocols, the hsTnI was analyzed on Access-2, using a commercial kits electrochemiluminescence assay (Beckman oulter, USA). H-FABP levels were determined by enzyme-linked immunosorbent assay (ELISA) using the commercial kit(Biovendor R&D, CZ) as described by the manufacturer's protocol.

Diagnostic outcomes were categorized as one of the following: ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI), unstable angina (UA), and non-cardiac chest pain (NCCP).

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software version 21.0. The data presented in number (N) and percentage (%). The two strategies/approaches namely DSA and DMA, were compared for their early rule in (high positive predictive value and specificity) and early rule out (high sensitivity and negative predictive value) capacity to diagnose AMI using sensitivity, specificity, positive predictive value, negative predictive value and area under curve (AUC). The optimum cut-offs for HFABP (>6.3 ng/ml) were taken from ROC analysis, defined as the biomarker level at which sum of sensitivity and specificity was highest to diagnose AMI[13]. P value <0.005 was considered as statistically significant. Sensitivity and specificity were calculated according to Gijsberts et al[14].

RESULTS

Out of the 80 enrolled patients with acute chest pain, 22 cases (27.5%) were confirmed to have AMI. Both acute chest pain patients (n=61(76.2%)) and AMI (n=19 (86.3%)) were more prevalent in males. Male: female ratio in AMI patients was significantly higher (6.3:1) than that in non-AMI patients (2.6:1; p<0.0001). The median age at presentation for AMI was 67.0 (IQR=15.25) years, which was significantly higher than acute chest pain patients (Median 57.5 years; IQR=21.75). NSTEMI was significantly associated with AMI in 11 patients (50 %) (p<0.0001). (Table 1)

Single biomarker assays

To achieve the maximum sum of sensitivity (86.4%) and specificity (94.2%), the optimal threshold (Cut-off) of H-FABP was 6.3 ng/ml as guided by ROC analysis. Upper Reference Limit (URL) for hsTnI at admission was 20.24 ng/L (as calculated from the non-AMI population; n=58) in our study.

Among the single biomarker assays, hsTnI and HFABP provide equal sensitivity (81.8%) to diagnose AMI at presentation (0 hour) as compared to cardiac troponin I (cTnI; sensitivity 63.6%). However, their specificity was much lower (89.6% for hsTnI and 86.2% for HFABP) in comparison to cTnI (100%) Fig 2A. The cut-off of cardiac troponin I was taken as 0.2 ng/ml as per manufacturer's instruction. On ROC analysis, hsTnI showed a slightly better area under the curve (0.87) than HFABP (0.84) to diagnose AMI at presentation (Table 2; Fig.1 A, B & C). The sensitivity of both the tests was 86.4% to diagnose AMI at presentation (Fig.2A & 2B).

DSA and DMA approach

When the same cut-offs as described above were applied in our two diagnostic algorithms, namely DSA and DMA, the sensitivity (95.5%), NPV (97.6%), and PPV (100%) was highest for DMA. At the same time, specificity was maximum (98.2%) for the DSA irrespective of the time of presentation since the onset of symptoms and ST elevation (Fig. 2A, 2B).

On the further assessment of the DMA approach, three findings were noteworthy: Firstly, if both markers were increased above their respective cut-offs i.e. H-FABP>6.3 ng/ml; hsTnI >20.24 ng/L (DMA: both positive; n=17), the PPV (100%) was maximium. Secondly, if neither of the markers were increased (both negative; n=41) above their respective cut-offs, NPV was 97.5% (Table 3A Fig. 2B). Thirdly, there was not

even a single AMI case where only one marker was raised if presentation time was > 3 hours. This indicates that if AMI occurs, both the markers would be elevated in 3 hours of onset of symptoms.

Since all the cases with STEMI were confirmed as AMI in our study group, we further focused on the patients with non ST elevation, where all the parameters of diagnostic performance remained same for DMA (specificity 86.5%, PPV 100%, NPV 97.6%) except sensitivity which drops down to 90.9%. It out performs DSA except for specificity (98.2%) (Fig. 2A, 2B). All the patients with both markers above cut-offs (DMA: both positive; n=6) were finally diagnosed as AMI (TP) while only one case (FN) was detected among patients with both the markers below cut-offs. Out of 10 DSA positive patients, only one was false positive while only two patients were diagnosed as AMI (FN) out of 59 patients with DSA (Table 3B).

Diagrammatic representation of rule-out/rule-in of AMI patients by applying DSA and DMA approach is summarized in Figure 3.

Table 1 **Descriptive characteristics of the study population**

	Suspected AMI patients (n= 80)		p value
	AMI (No); n=58	AMI (Yes); n=22	
Age Median (Years)(IQR)	67.0 (15.25)	57.5 (21.75)	
Gender Male Female (M:F)	 42 16 2.6:1	 19 3 6.3:1	<0.0001*
Diabetics	6	7	0.027*
Smokers	16	8	0.307
Hypertension	27	16	0.031*
Non-ST elevation	58	11	<0.001*
ST elevation MI (STEMI)	0	11	NA
Unstable Angina	14	0	NA
Non-Cardiac Chest Pain (NCCP)	44	0	NA

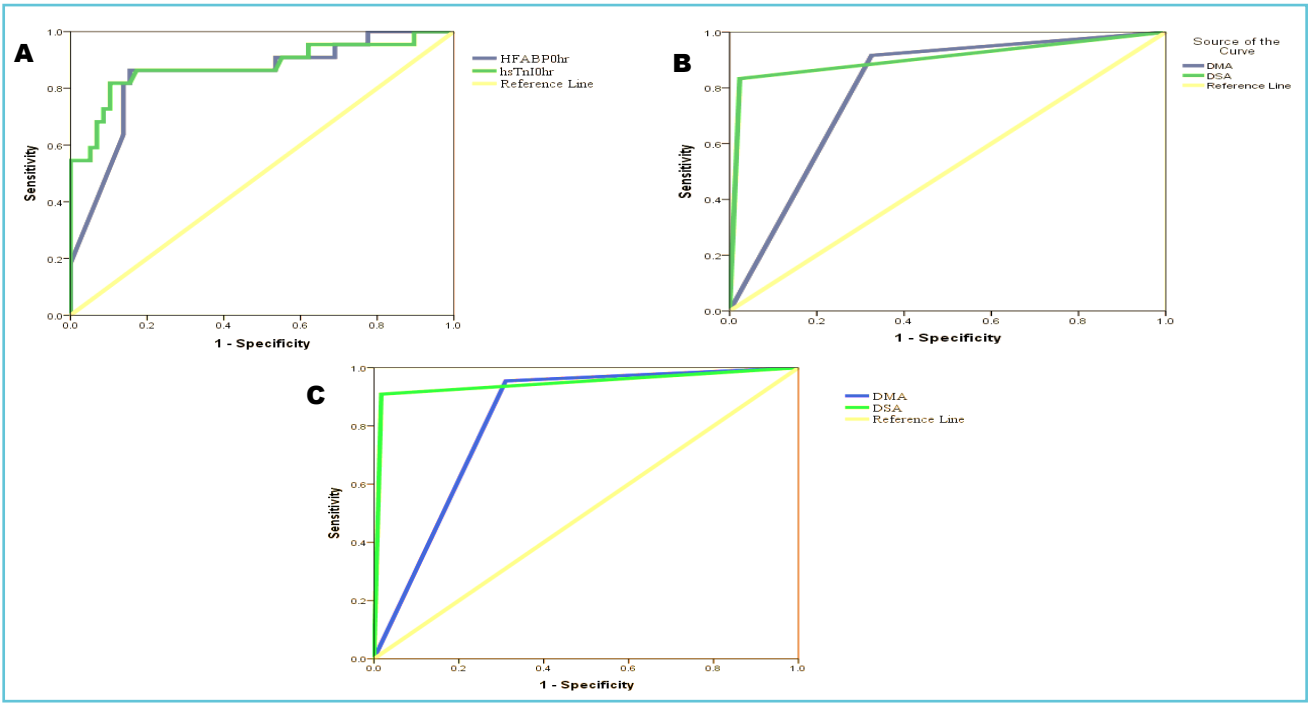
Abbreviation: IQR; Inter quartile range.

Table 2 ROC analysis of individual and combine approaches: within 3 hours of the onset of symptoms, within 3-6 hours of the onset of symptoms and overall

Variable(s)	AUC	SE	p-value	95% CI
Group A - Presentation within 3 hours of the onset of symptoms				
H-FABP>6.3 ng/ml at admission	0.73	0.10	0.03*	0.520-0.942
hsTnI>URL at admission	0.74	0.11	0.02*	0.521-0.967
H-FABP >6.3 ng/ml or hsTnI 20.24 ng/ L i.e. >URL at presentation (0 h)	0.78	0.07	0.008*	0.630-0.934
hsTnI at 0 & 3 hour (ESC 3-h guideline)	0.87	0.08	<0.0001*	0.712-1.000
Group B - Presentation within 3-6 hours of the onset of symptoms				
H-FABP>6.3 ng/ml at admission	0.85	0.09	0.11	0.661-1.000
hsTnI>URL at admission	0.93	0.07	0.05	0.793-1.000
H-FABP >6.3 ng/ml or hsTnI 20.24 ng/ L i.e. >URL at presentation (0 h)	0.86	0.09	0.10	0.681-1.000
hsTnI at 3 & 6 hour (ESC 3-h guideline)	1.00	0.00	0.02*	1.000-1.000
Group C - Overall performance (presentation combining both time groups)				
H-FABP>6.3 ng/ml at admission	0.84	0.05	<0.0001*	0.743-0.948
hsTnI>URL at admission	0.87	0.05	<0.0001*	0.774-980
H-FABP >6.3 ng/ml or hsTnI 20.24 ng/ L i.e. >URL at presentation (0 h)	0.82	0.04	<0.0001*	0.728-916
hsTnI at 0 & 3 hour (ESC 3-h guideline)	0.94	0.03	<0.0001*	0.873-1.000

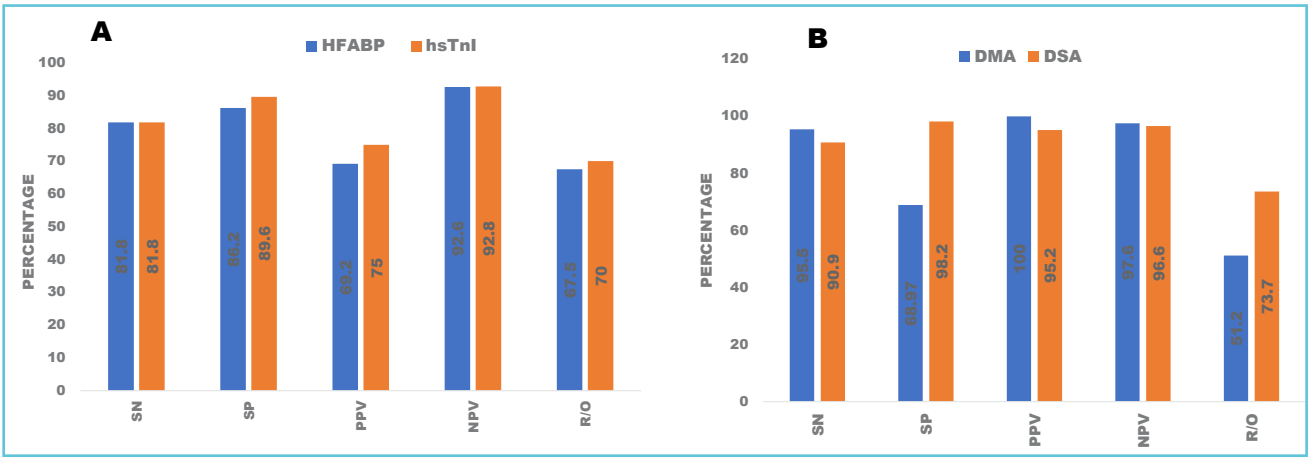
Abbreviations. H-FABP: Heart-type Fatty Acid Binding Protein, hsTnI: High sensitive Troponin-I, URL: Upper Reference Limit, CI: Confidence Interval, AUC: area under the curve<p 0.05 considered as significant.

Figure 1 ROC analysis of DMA & DSA approach: (A) Diagnostic performance of individual marker: hsTnI & HFABP at 0 hr; (B) Within 3 hours of onset of symptoms; (C) After 6 hours of the onset of symptoms



*Abbreviations: HFABP: Heart-type Fatty Acid Binding Protein, hsTnI: High sensitive Troponin-I, DMA: Dual Marker Approach, DSA: Double Sampling approach. *All SN, SP, PPV and NPV were 100% in STEMI cases not included.*

Figure 2 Overall performance (presentation combining both time groups; both NSTEMI and STEMI): (A) Comparison of diagnostic performances of HFABP & hsTnI at 0 hr; (B) Comparison of diagnostic performances of DMA & DSA approaches



*Abbreviations: HFABP: Heart-type Fatty Acid Binding Protein, hsTnI: High sensitive Troponin-I, DMA: Dual Marker Approach, DSA: Double Sampling approach, SN: Sensitivity, SP: Specificity, PPV: Positive Predictive Values, NPV: Negative Predictive Values, R/O: Ruled out patient.*All SN, SP, PPV and NPV were 100% in STEMI cases (excluded in the graph).*

Table 3A **Comparison of diagnostic performance using different approaches of marker individually and in combination (in all studied cases)**

Approach			AMI	No AMI
cTnI alone	c TnI>0.2 ng/ml	Positive	15	01
		Negative	07	57
H-FABP alone	H-FABP>6.3 ng/ml at admission	Positive	18	08
		Negative	04	50
hsTnI alone	hsTnI>URL at admission	Positive	18	06
		Negative	04	52
DMA	H-FABP >6.3 ng/ml or hsTnI 20.24 ng/L i.e. >URL at presentation (0 hour)	Both Positive	17(TP)	00(FP)
		Either Positive	04(TP)	06(FP)
		Both Negative	01(FN)	40(FN)
DSA	hsTnI at 0 & 3 hour (ESC 3-hour guideline)	Positive	20	01
		Negative	02	57

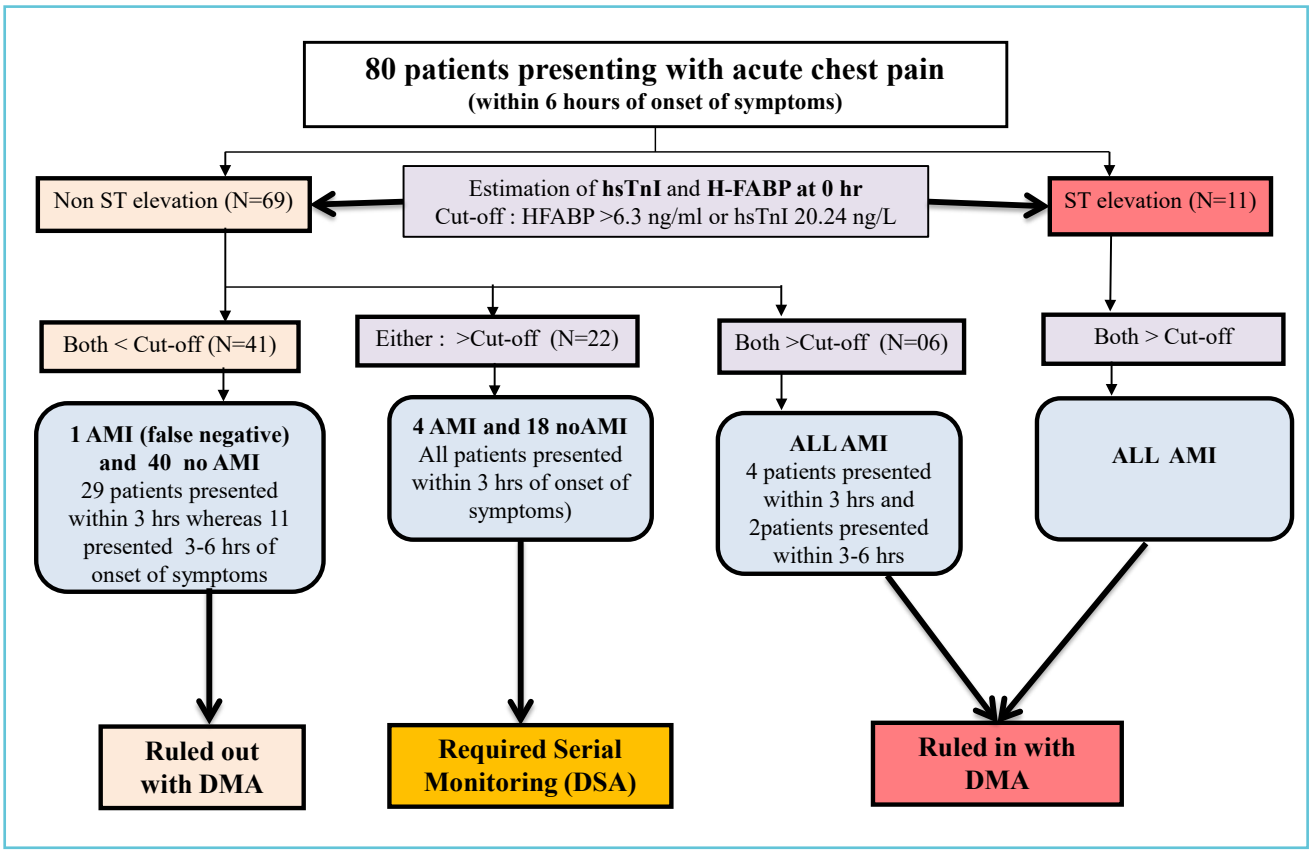
Table 3B **Comparison of diagnostic performance using different approaches of marker individually and in combination (only in patients with non ST elevation)**

Approach			AMI	No AMI
cTnI alone	cTnI >0.2 ng/ml	Positive	04	01
		Negative	07	57
H-FABP alone	H-FABP>6.3 ng/ml at admission	Positive	07	08
		Negative	04	50

hsTnI alone	hsTnI>URL at admission	Positive	07	06
		Negative	04	52
DMA	H-FABP >6.3 ng/ml or hsTnI 20.24 ng/L i.e. >URL at presentation (0 hour)	Both Positive	06(TP)	00(FP)
		Either Positive	04(TP)	06(FP)
		Both Negative	01(FN)	40(TN)
DSA	hsTnI at 0 & 3 hour (ESC 3-hour guideline)	Positive	09 (TP)	01 (FP)
		Negative	02 (FN)	57 (TN)

Abbreviations: H-FABP: Heart-type Fatty Acid Binding Protein, hsTnI: High sensitive Troponin-I, DMA: Dual Marker Approach, DSA: Double Sampling approach, URL: Upper Reference Limit, ESC: European Society of Cardiology, AMI: Acute Myocardial Infarction, TP: True Positive, FP: False Positive, TN:True Negative, FN: False Negative.

Figure 3 **Diagrammatic representation of rule-out/rule-in of AMI patients by applying DSA & DMA approach**



Abbreviations: HFABP: Heart-type Fatty Acid Binding Protein, hsTnI: High sensitive Troponin-I, AMI: Acute Myocardial Infarction, DMA: Dual Marker Approach, DSA: Double Sampling approach.

DISCUSSION

Diabetes mellitus and hypertension are well-known risk factors[14,15] for AMI, consistent with our findings (Table 1). Though smokers prevalence was not higher in our AMI patients, it has been well associated with other causes of cardiac/non-cardiac chest pain (NCCP), evident from our data. The total prevalence of smoking among our study subjects was 30 % (n=24).

Our study took the cut-off for H-FABP as 6.3 ng/ml from ROC analysis. Certain single-center Indian studies[15-17] have also described very similar thresholds (6.3, 6.4, and 6.32 ng/ml), respectively. Similar results ranging from 5.0 to 7.0 ng/ml have also been published by others[18-21], these slight variations may be due to the difference in the sampling timing from the onset of symptoms, methodology used, demographics, and ethnicity. Our findings agree with the opinion of McCann et al., who declared that assessment of H-FABP within the first 4 h of symptom onset is superior to that of cardiac Troponin T (cTnT) for the detection of AMI[21].

In this study, we compared the performance of hsTnI or H-FABP alone as well as their combinations (DMA) at admission (0 hrs) along with the DSA (using hsTnI at 0 hr and 3-hr sampling) to accurately rule in/rule out AMI in eighty acute chest pain patients in an emergency settings.

Our results showed that among patients with non-ST-elevation, the PPV of DMA was 100% if both markers were elevated. It efficiently rules out 51.2% cases (sensitivity 95.5%, NPV 97.6%; only one false negative) for AMI. None of the biomarkers (cTnI, hsTnI, or HFABP) at presentation was sufficient for a reliable rule in/ rule out of AMI when used alone.

On serial monitoring of hsTnI at 0 hr and 3 hr interval (DSA), both sensitivity (90.9%) and specificity (98.2%) and area under the curve (AUC) are significantly enhanced in comparison to when URL of hsTnI or cTnI (at cut-off of 0.2 ng/ml) were used alone at presentation (0 hour). A single negative cTnI test is not sufficient to disregard the presence of AMI because of its low sensitivity in the first 3 h of chest pain onset as per previous reports[22, 23].

In ROC analysis our data revealed that area under curve (AUC) for HFABP was 0.84 (95% Confidence Interval; 0.743-0.948) to diagnose AMI at presentation. These findings were parallel to previous studies 0.830 (95% CI; 0.770–0.890)[20] and 0.800 (95% CI; 0.760-0.840)[22] while slightly deviated from findings of Reddy et al[17] i.e. 0.728(95% CI; 0.622-0.817) and Ruff et al[24] i.e. 0.780 (95% CI; 0.720-0.840). Similarly, area under curve (AUC) for hsTnI was 0.87 (95% CI; 0.774-0.980) in our study which was consistent with Eggers et al[22] 0.840 (95% CI; 0.800-0.880) while Kellens et al[20] and Ruff et al[24] reported it as 0.790 (95% CI of 0.73–0.85) and 0.956 (95% CI; 0.930-0.990) respectively. These minor deviations might be due to heterogenity of presentation since the onset of symptoms, methodology, instrumentation and ethnicity.Eleven (11) subjects presented with ST-elevation (n=11) on ECG; all had an elevation of both hsTnI and HFABP at 0 h sample and were later confirmed as AMI cases in our study group. Thus, both the biomarkers may be used reliably as supportive evidence in such cases to rule out any subjective variation. We further focused on non-ST-elevation subjects where the role of DMA seems pertinent with PPV same as that in overall study population (presenting with or without ST elevation). Though the sensitivity decreased slightly, DMA same proportion of patients owing to same NPV.

Our results for DSA for the overall patient population (irrespective of ST-elevation and time of presentation; Table-3A) complied with that depicted by Pickering et al.[5] who noted sensitivity (95.4%), specificity (96.5%), PPV (91.3%) and NPV (98.2%).

When both markers were elevated, the diagnosis of AMI was made irrespective of the presentation time since the onset of symptoms. Among cases (n=41) with both markers below the cut-off values, only one false negative case was found.

The history of the false-negative patient was further investigated in detail. He was a 68-year-old male who lived nearby and reached the hospital within 20 minutes of developing acute chest pain. His pain was relieved by itself within 30 minutes and recurred after 15 minutes of the first episode. On the first sample taken subsequently, i.e., at admission ('0' hour), both hsTnI (2.3 ng/L) and H-FABP (1.05 ng/ml) were very low while hsTnI was raised to 14.0 ng/L at 3 hours post-admission sample. Thus, this type of case (with very early presentation) may not be ruled out in with single sample since it is also possible that ischemia might have started after the first sampling (as suggested by clinical history). The role of careful history taking and a high index of suspicion must not be overlooked in such cases, which may occur in clinical practice.

Our study focused on the strategic evaluation of different approaches for effective rule out/rules in for the diagnosis of AMI. It was done on a limited population at a single center. A multicentric study with a large number of subjects, preferably with a multimarker approach, is warranted for further evaluation.

Further, ELISA based HFABP estimation is time consuming though robust. Commercial kits on fully automated analyzers are recently available claiming remarkable analytical sensitivity. In future trials, it may be included in DMA algorithm to get rapid result and enhance practical utility in emergency settings.

CONCLUSION

A single marker assay (hsTnI or HFABP) is associated with false positives as well as false negatives. The proposed dual marker approach using H-FABP in tandem with hsTnI enhances sensitivity, positive and negative predictive values. With the use of the proposed DMA, 58/80 (72.5%) patients with acute chest pain were ruled in/ruled out for AMI at the time of their presentation itself, while the remaining 27.5% of patients still required serial monitoring (DSA) for confirmation.

❖❖❖❖❖

Acknowlegment

Authors sincerely acknowledge all the patients enrolled in this study for their consent to participate in the research work as study subject.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Study is a part of MD thesis.

Authors' contribution

Dr. Manish Raj Kulshrestha: Conceptualization and drafting of manuscript graphs and tables preparation.

Dr. Apoorv Raj: Sample collection, history & data collection of enrolled patients.

Dr. Vandana Tiwari: Conceptualisation, Experimentation, Manuscript writing, Result analysis and manuscript reviewing and editing.

Dr. Bhuwan Chandra Tiwari: Patient enrollment, Clinical evaluation and data collection.

Dr. Ashish Jha: Patient enrollment and Clinical evaluation.

Dr. Subrat Chandra: Data collection and manuscript formatting.

❖❖❖❖❖

REFERENCES

1. Alhashemi JA. Diagnostic accuracy of a bedside qualitative immunochromatographic test for acute myocardial infarction. The American journal of emergency medicine. 2006 Mar 1;24(2):149-55.

2. PyAti AK, Devaranavadagi BB, SAjjAnnAr SL, et al. Heart-type fatty acid binding protein: a better cardiac biomarker than CK-MB and myoglobin in the early diagnosis of acute myocardial infarction. Journal of Clinical Diagnosis and Research: 2015. 9(10):BC08.

3. Deshpande A, Birnbaum Y. ST-segment elevation: distinguishing ST elevation myocardial infarction from ST elevation secondary to nonischemic etiologies. World Journal of Cardiology: 2014. 6(10):1067.

4. Meng QH, Zhu S, Booth C, Stevens L, Bertsch B, Qureshi M, Kalra J. Impact of the cardiac troponin testing algorithm on excessive and inappropriate troponin test requests. American journal of clinical pathology. 2006 Aug 1;126(2):195-9.

5. Pickering JW, Young JM, George PM, Pemberton CJ, Watson A, Aldous SJ, Verryt T, Troughton RW, Richards AM, Apple FS, Than MP. Early kinetic profiles of troponin I and T measured by high-sensitivity assays in patients with myocardial infarction. Clinica Chimica Acta. 2020 Jun 1;505:15-25.

6. Danese E, Montagnana M. An historical approach to the diagnostic biomarkers of acute coronary syndrome. Annals of translational medicine. 2016 May;4(10).

7. Apple FS, Collinson PO, IFCC Task Force on Clinical Applications of Cardiac Biomarkers. Analytical characteristics of high-sensitivity cardiac troponin assays. Clinical chemistry. 2012 Jan 1;58(1):54-61.

8. Lippi G. Novel troponin immunoassay for early ACS rule-out. Nature Reviews Cardiology. 2016 Jan;13(1):9-10.

9. Druey S, Wildi K, Twerenbold R, Jaeger C, Reichlin T, Haaf P, Gimenez MR, Puelacher C, Wagener M, Radosavac M, Honegger U. Early rule-out and rule-in of myocardial infarction using sensitive cardiac Troponin I. International Journal of Cardiology. 2015 Sep 15;195:163-70.

10. Wu AH, Christenson RH. Analytical and assay issues for use of cardiac troponin testing for risk stratification in primary care. Clinical Biochemistry, 2013. 46(12):969-78.

11. Wu AH, Feng YJ, Contois JH, Pervaiz S. Comparison of myoglobin, creatine kinase-MB, and cardiac troponin I for diagnosis of acute myocardial infarction. Annals of Clinical & Laboratory Science. 1996 Jul 1;26(4):291-300.

12. Thygesen K, Alpert JS, Jaffe AS et al. Executive Group on behalf of the Joint European Society of Cardiology (ESC)/American College of Cardiology (ACC)/American Heart Association (AHA)/World Heart Federation (WHF) Task Force for the Universal Definition of Myocardial Infarction. Fourth Universal Definition of Myocardial Infarction. Circulation, 2018. 138(20):e618-51.

13. Gijsberts CM, Seneviratna A, Bank IE et al. The ethnicity-specific association of biomarkers with the angiographic severity of coronary artery disease. Netherlands Heart Journal, 2016. 24(3):188-98.

14. Jensen G, Nyboe J, Appleyard M, Schnohr P. Risk factors for acute myocardial infarction in Copenhagen II: Smoking, alcohol intake, physical activity, obesity, oral contraception, diabetes, lipids, and blood pressure. European heart journal. 1991 Mar 1;12(3):298-308.

15. Tokuda Y. Risk factors for acute myocardial infarction among Okinawans. The Journal of nutrition, health & aging. 2005 Jul 1;9(4):272-6.

16. Vupputuri A, Sekhar S, Krishnan S et al. Heart-type fatty acid-binding protein (H-FABP) as an early diagnostic biomarker in patients with acute chest pain. Indian Heart Journal, 2015. 67(6):538-42.

17. Reddy LL, Shah SA, Dherai AJ, Ponde CK, Ashavaid TF. Troponin T and heart type fatty acid binding protein (h-Fabp) as biomarkers in patients presenting with chest pain. Indian Journal of Clinical Biochemistry. 2016 Jan;31(1):87-92.

18. Liao J, Chan CP, Cheung YC, Lu JH, Luo Y, Cautherley GW, Glatz JF, Renneberg R. Human heart-type fatty acid-binding protein for on-site diagnosis of early acute myocardial infarction. International journal of cardiology. 2009 Apr 17;133(3):420-3.

19. Mad P, Domanovits H, Fazelnia C, Stiassny K, Russmüller G, Cseh A, Sodeck G, Binder T, Christ G, Szekeres T, Laggner A. Human heart-type fatty-acid-binding protein as a point-of-care test in the early diagnosis of acute myocardial infarction. Journal of the Association of Physicians. 2007 Apr 1;100(4):203-10.

20. Kellens S, Verbrugge FH, Vanmechelen M, Grieten L, Van Lierde J, Dens J, Vrolix M, Vandervoort P. Point-of-care heart-type fatty acid binding protein versus high-sensitivity troponin T testing in emergency patients at high risk for acute coronary syndrome. European Heart Journal: Acute Cardiovascular Care. 2016 Apr 1;5(2):177-84.

21. McCann CJ, Glover BM, Menown IB, Moore MJ, McEneny J, Owens CG, Smith B, Sharpe PC, Young IS, Adgey JA. Prognostic value of a multimarker approach for patients presenting to hospital with acute chest pain. The American journal of cardiology. 2009 Jan 1;103(1):22-8.

22. Eggers KM, Venge P, Lindahl B. High-sensitive cardiac troponin T outperforms novel diagnostic biomarkers in patients with acute chest pain. Clinica chimica acta. 2012 Jul 11;413(13-14):1135-40.

23. Cubranic Z, Madzar Z, Matijevic S, Dvornik S, Fisic E, Tomulic V, Kunisek J, Laskarin G, Kardum I, Zaputovic L. Diagnostic accuracy of heart fatty acid binding protein (H-FABP) and glycogen phosphorylase isoenzyme BB (GPBB) in diagnosis of acute myocardial infarction in patients with acute coronary syndrome. Biochemia Medica. 2012 Jun 15;22(2):225-36.

24. Ruff CT, Bonaca MP, Kosowsky JM, Conrad MJ, Murphy SA, Jarolim P, Donahoe SM, O'Donoghue ML, Morrow DA. Evaluation of the diagnostic performance of heart-type fatty acid binding protein in the BWH-TIMI ED chest pain study. Journal of thrombosis and thrombolysis. 2013 Nov;36(4): 361-7.

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

This is a Platinum Open Access Journal distributed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Importance of observing the progress curve during enzyme assay in an automated clinical chemistry analyzer: a case study

Vivek Pant

Department of Clinical Biochemistry, Samyak Diagnostic Pvt. Ltd, Kathmandu, Nepal

ARTICLE INFO

Corresponding author:
Dr. Vivek Pant
Department of Clinical Biochemistry
Samyak Diagnostic Pvt. Ltd
Kathmandu
Nepal
E-mail: drvpant@gmail.com

Key words:
enzyme, substrate exhaustion, sample dilution, progress curve

ABSTRACT

In the case presented here the reported result for total creatine phosphokinase led to the physician calling for report confirmation. The repeated test result was in keeping with the clinical picture and thus the previous erroneous result was amended. The incorrect result from auto analyzer was identified as failure to run the sample in dilution after instrumental flagging of possible substrate exhaustion evidenced by erroneous progress curve. A frequent reason for nonlinear progress curves is the presence of excess enzyme which can be easily misinterpreted as lower enzyme activity in a provided sample. Careful inspection of progress curve and predilution of sample in anticipated cases could avoid erroneous result.

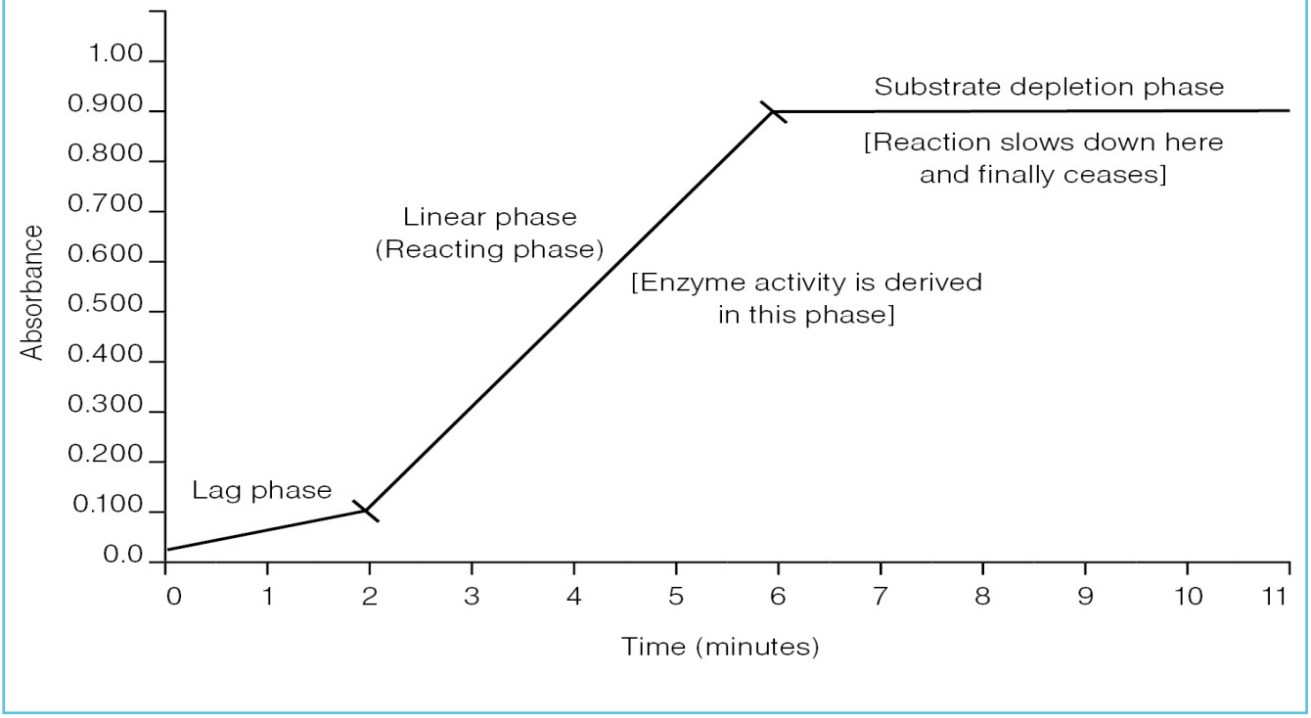
INTRODUCTION

Concentration of enzymes is very low in plasma or body fluid and cannot be directly measured as in case of other analytes such as glucose or total protein. Thus, enzymes are indirectly measured by their catalytic activity which is proportional to their concentration.

The principal of kinetic assay is that, if the concentration of the substrate (S) is sufficiently high in comparison to enzyme (E) then the rate of reaction will be proportional to the concentration of the enzyme. Therefore, the amount of product (P) formed in a given period of time would be proportional to the amount of active enzyme present, with all other factors remaining constant. To determine the reaction velocity and for evaluating the enzyme activity, the plot of absorbance against time is required. This plot also known as progress curve permits the detection of erroneous influences and the control of the reaction course. (Figure 1) A catalyzed reaction must initially follow a linear relationship, from which its velocity and eventually enzyme activity is derived. Due to depletion of substrates during the later progression the reaction slows down and finally ceases. (Figure 1)

The Michaelis-Menten equation provides the "activity" of the enzyme (1). The rate of reaction when the enzyme is saturated with substrate is the maximum rate of reaction, Vmax. The relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate. This is usually expressed as the Km (Michaelis constant) of the enzyme, an inverse measure of affinity. For practical purposes, Km is the concentration of substrate which permits the enzyme to achieve half Vmax. An enzyme with a high Km has a low affinity for its substrate, and requires a greater concentration of substrate to achieve Vmax. This means that the concentration of substrate must be high enough to ensure that the enzyme

Figure 1 Normal progress curve of a catalyzed reaction



is acting at Vmax (1). In practice, it is usual to use a concentration of substrate about 10 – 20 fold higher than the Km in order to determine the activity of an enzyme in a sample (2). Majority of enzymes have Km values in order of 10^{-5} to 10^{-3} mol/L.

The present report emphasizes the importance of observing the plot of absorbance against time to identify the linearity slope, while performing creatine phosphokinase (CK) enzyme assay.

CK is a large protein made up of two distinct polypeptide subunits, M and B (3). Three isoenzymes of CK are found in human tissue: CK-MM (skeletal muscle), CK-MB (cardiac muscle), and CK-BB (brain). CK catalyses the reversible transfer of energy-rich phosphate from creatine phosphate to adenosine diphosphate (ADP), thus forming adenosine triphosphate (ATP) that is utilized by muscle myofibrils during its contraction. The molecular structure of CK prevents it from being released from the host tissue into the bloodstream, except in muscle membrane injury. Serum concentrations of CK is therefore increased when muscle is damaged after strenuous physical activity, trauma, crush injury, myositis, muscular dystrophy, intramuscular injection, convulsions, myocardial infarction, malignant hyperthermia and drugs such as aminophyline and succinylcholine (4). CK measurement in serum is the gold standard test to detect and monitor skeletal muscle diseases and damage. Thus, it is essential to mention the clinical diagnosis while ordering this test.

CLINICAL-DIAGNOSTIC CASE

We received a serum sample from a 48 years male collected at a remote hospital, for analysis of total creatinine kinase. The sample was analyzed using RX Imola auto-analyzer (Randox Laboratories Ltd). Daily maintenance for this auto analyzer was conducted and internal quality control sample from Bio-Rad was run which were found to be within the acceptable range. The patient result of 12 U/L [24 – 195 U/L] was reported. However, we received a call from attending physician stating that they had expected higher value for total CK and requested to repeat the assay. The provisional diagnosis of patient was acute kidney injury secondary to lacerated wound over left lower limb.

Careful inspection for potential source of error in all analytical steps and reanalyzing the serum sample was planned.

Preanalytical factors affecting CK are age, race, muscle mass, physical activity, medication and hypothyroidism. Only severe hemolysis affects CK since red blood cells (RBC) have no CK. Release of enzymes and intermediates from RBC such as adenylate kinase (AK), ATP and glucose-6-phosphate (G-6-P) may inhibit CK in severe hemolysis. No preanalytical error was identified in the index case.

The reagent, calibrator, internal quality control graphs and instrument were checked to identify presence of any analytical error. Total CK is performed in serum or heparinised plasma. CK enzyme activity is easily inhibited by factors both in vivo and in vitro, thus automated assay includes factors to preserve enzyme action and ensure accurate measurement. For example, the reagent contains N-acetyl cysteine (NAC) to reactivate sulfhydryl group in the centre of CK which is prone to rapid oxidation with loss of CK activity. The diadenosine pentaphosphate and adenosine monophosphate (AMP) is added to inhibit adenylate kinase, which may be present in platelets of patient with liver disease. Also, the reagent contains magnesium to complex with ADP and ATP.

The enzymatic reaction catalyzed by CK is reversible, however all commercial assays for CK are based on creatine-to-creatine phosphate reaction as it proceeds six times faster than creatine phosphate to creatinine. ATP liberated

phosphorylates glucose to glucose-6-phosphate. Glucose-6- phosphate is oxidized to 6-phosphogluconate, reducing Nicotinamide adenine dinucleotide phosphate (NADP) to NADPH in presence of glucose 6-phosphate dehydrogenase (G6PD). The rate of increase in NADPH absorbance at 340nm is directly proportional to the activity of CK present in serum. The difference between commercial assays is the use of buffer (imidazole or Tris) and source of G6PD (yeast or from bacterium) that however does not interfere with the assay. The reagent was in standard condition and no error was identified during sampling.

The internal quality control for CK in the past ten days was within 1SD and calibration was up to date. There was no recent maintenance of instrument and no issues with other parameters. The technician was however recently employed. The reported result was inspected in the instrument, and it was found that the assay was repeated automatically with two results where the first one indicated error with flagging as "E" and second one was 12 U/L. Thus, the technician reported the result as 12 U/L through laboratory information system (LIS). The inspection of progress curve indicated an erroneous result.

The normal progress curve for each analyte could be inspected in the auto analyzer, from the stored data generated by sampling of the quality control for that specific analyte. Any deviation from this normal progress curve should alert the technician. Thus, analytical error was identified and repetition of the test from stored sample was done.

We anticipated higher value for total CK from the provisional diagnosis. Thus, we diluted the sample and the total CK was 150891.98 U/L after 1:100 dilutions. The result was informed to the physician and the initial erroneous result was amended.

DISCUSSION

The basic principle of kinetic enzyme assay is that when the substrate concentration is very high the reaction rate is independent of substrate concentration. (1) Under the normal circumstances, the concentration of the substrate contained in a reagent is large enough to meet the actual testing needs of most clinical samples. Enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatine kinase (CK) may be released in very large quantity in certain clinical conditions affecting liver, heart, muscle or other organs. During such a clinical situation, the enzyme activity exceeds the linear range of the kit which no longer maintains the reaction at zero order and the reported results are far below the actual concentration.

In practice the reaction rate is not constant with time. There is an initial (i) lag phase (with very little change per unit of time), then comes (ii) linear phase of constant change per unit of time and then finally (iii) phase of substrate exhaustion with very little change. In order to obtain the best result we should have a long linear phase and assay should be stopped before substrate exhaustion takes place. Too much of enzyme will consume all the substrate immediately and no more reaction can be observed when the recording is started. Such a situation is easily misinterpreted as lack of activity and the reported enzyme concentration is very low. The substrate in the above mentioned reaction is creatine phosphate. Creatine phosphate gets consumed by very high concentration of CK which was present in the serum sample before the kinetic measurement actually gets initiated. The whole substrate gets consumed within the lag phase which results in falsely low values.

This effect is also a recognized limitation in immunoassay, where it is known as prozone effect which occurs when the number of analyte

molecules exceeds the number of antibody binding sites. There are numerous reports of this effect in wide ranges of analytes as shown in Table 1. Many modern immunoassays have been formulated for automated dilution in cases of prozone effect.

The clinical performance of the biochemistry analyzer is affected by many factors such as the reagents, the integration and settings, the traceability system of the manufacturer, daily calibration and quality control operation and maintenance of the instrument. When choosing an analyzer, the laboratory should pay special attention to the substrate depletion limit of the kinetic method. Different laboratories may have different ranges of substrate depletion due to variable detection system, sample dilution ratios and parameter settings. Automatic dilution of sample in cases of substrate exhaustion was lacking in our analyzer and the recently employed technician failed to recognize the instrumental flagging for erroneous result which led to wrong reporting initially.

The erroneous result from auto analyzers could be avoided by observing the plot of absorbance against time to identify the linearity slope since various analyzers usually present this figure. Those instruments which display the absorbance and continue to display the changing absorbance with the chemical reaction are considered superior for kinetic assays. It can monitor the rate of reaction depending on the high or low concentration of enzyme and also help in detecting substrate exhaustion along with timings. If we can anticipate higher values of the enzyme (beyond the linearity range) we can predilute the sample and then run the test so as to avoid repetition. For example: an icteric sample may have higher values of transaminase and if clinical history of trauma or lacerated wound is provided, then the sample may have high CK value (Table 2). This case therefore also

Table 1 Reports of clinical assay demonstrating prozone effect

Biochemical assay	Clinical diagnosis	Reference
Prolactin	Prolactinoma	5,6
β-hCG	Advanced molar Pregnancy	7
Calcitonin	Metastatic medullary thyroid carcinoma	8
Prostate specific antigen	Advanced prostate cancer	9
17-hydroxyprogesterone	21-hydroxylase deficiency	10
CA-125	Ovarian carcinoma	11
Alpha fetoprotein	Hepatoblastoma	12
IgE	Atopic dermatitis	13

Table 2 Biochemical parameters where the reading of progress curves is important

Analyte	Suggestive history or clinical condition where predilution of sample is helpful
ALP	Icteric sample, Obstructive jaundice, Bone tumor
Amylase and Lipase	Lipemic sample, Patient under evaluation for pain abdomen, History of alcohol intake
CK	Muscle trauma, Crush Injury, muscular dystrophy
CK-MB	Electrocardiogram changes, Myocardial infarction
ALT	Icteric sample, Liver disease
AST	Icteric sample, Liver disease
Gamma GT	Icteric sample, Liver disease
LDH	Cancer, Anemia, Myocardial infarction
Myoglobin	Muscle trauma, Rhabdomyolysis

highlights the importance of mentioning the provisional diagnosis while ordering the test.

LEARNING POINTS

- A frequent reason for nonlinear progress curves is the presence of excess high enzyme level which is easily misinterpreted as lack of enzyme activity in the reported result.
- Careful inspection of progress curve and predilution of sample in anticipated cases could avoid erroneous result.

REFERENCES

1. Cornish-Bowden A. Fundamentals of enzyme kinetics. John Wiley & Sons; 2013 Feb 22.

2. Panteghini M, Bais R. Tietz text book of clinical chemistry and molecular diagnostics. 5th edn. Philadelphia: Saunders; 2012. Serum Enzymes In: Burtis CA, Ashwood ER, Bruns DE editors; pp. 565–98.

3. Schneider, Carole M., et al. "Effects of physical activity on creatine phosphokinase and the isoenzyme creatine kinase–MB." Annals of emergency medicine 25.4 (1995): 520-524.

4. Lott JA, Stang JM. Differential diagnosis of patients with abnormal serum creatine kinase isoenzymes. Clinics in laboratory medicine. 1989 Dec 1; 9(4):627-42.

5. Frieze TW, Mong DP, Koops MK. "Hook effect" in prolactinomas: case report and review of literature. Endocrine practice. 2002 Jul 1;8(4):296-303.

6. Kim SY, Park CG, Choi YJ, Hong ES, Kim SW, Shin CS, Jang HC, Kim SY, Cho BY, Lee HK. High-dose hook effect in patients with macroprolactinoma. Journal of korean society of endocrinology. 2005 Apr 1;20(2):148-53.

7. Winder AD, Mora AS, Berry E, Lurain JR. The "hook effect" causing a negative pregnancy test in a patient with an advanced molar pregnancy. Gynecologic oncology reports. 2017 Aug;21:34.

8. Leboeuf R, Langlois MF, Martin M, Ahnadi CE, Fink GD. "Hook effect" in calcitonin immunoradiometric assay in patients with metastatic medullary thyroid carcinoma: case report and review of the literature. The journal of clinical endocrinology & metabolism. 2006 Feb 1;91(2):361-4.

9. Akamatsu S, Tsukazaki H, Inoue K, Nishio Y. Advanced prostate cancer with extremely low prostate-specific antigen value at diagnosis: An example of high dose hook effect. International journal of urology. 2006 Jul;13(7):1025-7.

10. Parlak M, Ellidağ HY, Türkkahraman D. High-dose hook effect in 17-hydroxyprogesterone assay in a patient with 21-hydroxylase deficiency. Journal of clinical research in pediatric endocrinology. 2015 Dec;7(4):329.

11. Pesce MA. " High-dose hook effect" with the Centocor CA 125 assay. Clinical chemistry. 1993 Jun 1;39(6): 1347.

12. Jassam N, Jones CM, Briscoe T, Horner JH. The hook effect: a need for constant vigilance. Annals of clinical biochemistry. 2006 Jul 1;43(4):314-7.

13. Saryan JA, Garrett PE, Kurtz SR. Failure to detect extremely high levels of serum IgE with an immunoradiometric assay. Annals of allergy. 1989 Oct 1;63(4): 322-4.

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

This is a Platinum Open Access Journal distributed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Secondary hemophagocytic lymphohistiocytosis – a common ramification of different diseases

Janani Gnanasambandam, Sangeetha Geminiganesan, Vairan Vilvanathan, Shuba Sankaranarayanan, Ramachandran Padmanabhan

Department of Paediatric Medicine, Sri Ramachandra Institute of Higher Education and Research, Chennai, India

ARTICLE INFO

Corresponding author:
Sangeetha Geminiganesan
Department of Paediatric Medicine
Sri Ramachandra Institute
of Higher Education and Research
Chennai
India
E-mail: sangeethaperungo@gmail.com

Key words:
hemophagocytic lymphohostiocytosis, typhoid fever, dengue and hyperinflammation

ABSTRACT

Hemophagocytic lymphohistiocytosis is a rare and potentially fatal disorder caused by immune dysregulation. It can occur as a primary genetic disease or secondarily due to various causes including infections, malignancies or autoimmune diseases. In this case report, we present two cases of Hemophagocytic lymphohistiocytosis which were secondary to typhoid and dengue fever. While primary disease occurs predominantly in infants, secondary hemophagocytic lymphohistiocytosis can occur in any age group. Both primary and secondary hemophagocytic lymphohistiocytosis are characterised by fever, hepatosplenomegaly, pancytopenia and multiorgan dysfunction. But unusual persistence of fever and other organ involvement should need further workup for hemophagocytic lymphohistiocytosis. Secondary hemophagocytic lymphohistiocytosis may resolve on treating the underlying disorder. But severe cases need treatment with immunosuppressive/immunomodulation therapy to

prevent morbidity. Early clinical suspicion, prompt diagnosis and treatment of hemophagocytic lymphohistiocytosis are essential to prevent deleterious effects to health.

❖❖❖❖❖

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH), also identified as hemophagocytic syndrome, is an aggressive, life-threatening hematological syndrome characterised by unregulated immune activation resulting in malignant inflammation and multi-organ failure (1). The reported annual incidence of HLH is approximately 1.2 per million individuals. The mortality rate is very high, 95% if left untreated. Based on the underlying etiology, HLH is classified into primary (genetic) and secondary (acquired), both of which clinically manifest as acute or subacute febrile illness with hepatosplenomegaly, bi- or trilineage cytopenia, hypertriglyceridemia and hypofibrinogenemia. Inherited genetic mutations play a major role in primary HLH, whereas secondary HLH is commonly associated with infections, autoimmune disorders or malignancies (2). We report here the cases of two children who presented with acquired HLH secondary to enteric fever and dengue.

CLINICAL CASE DESCRIPTION

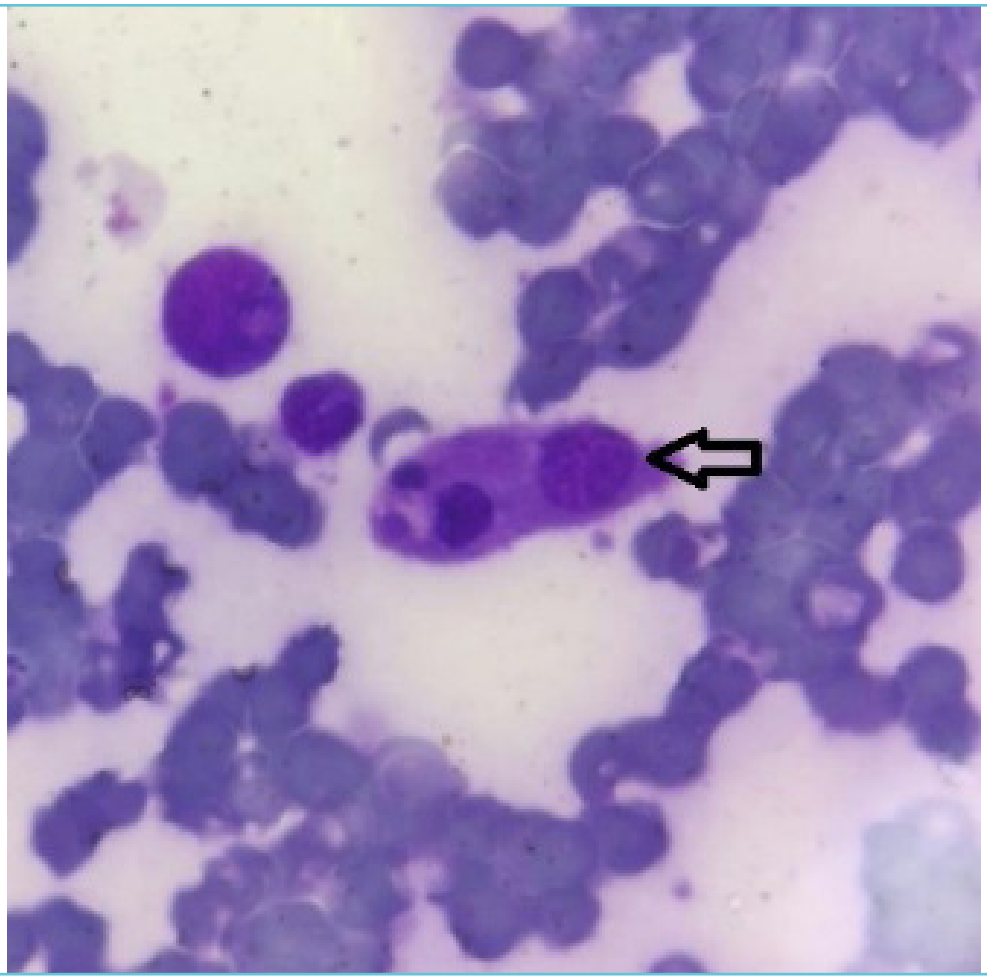
Case 1

A 5-year-old girl presented with high grade fever, abdominal pain and loose stools for 5 days, associated with reduced activity and poor oral intake for 2 days. There was history of fast breathing and reduced frequency of urine for a day. No history of vomiting, rash, joint pain, jaundice or edema. On examination, the child had tachypnea (respiratory rate - 87/min), tachycardia (heart rate - 160/min), cool peripheries, peripheral pulses were feeble and blood pressure was 100/70 mmHg. Her liver was enlarged to 4 cm below right costal margin and spleen was 3 cm below left costal margin. Both were soft in consistency and non-tender. There were no added sounds on auscultation. Provisional diagnosis of sepsis with compensated shock was made. Differential diagnosis was pneumonia, typhoid fever, scrub typhus and dengue fever.

Management and outcome

Baseline investigations revealed pancytopenia (total WBC count-3820 cells/mm^3, hemoglobin - 8.4 g/dl, platelets – 39×10^9/L), elevated liver enzymes (SGOT - 368 U/L, SGPT - 167 U/L), C reactive protein 86 mg/dL and normal renal parameters. Thorax ultrasound showed right-sided pleural effusion with bilateral B lines. Treatment was commenced with high flow oxygen by nasal cannula with FiO_2 of 30% and flow of 24L/min and was resuscitated with fluid bolus 20 ml/kg, normal saline. Intravenous ceftriaxone was started after drawing blood for culture. In view of high-grade fever which did not touch the baseline, increasing hepatosplenomegaly and worsening pancytopenia on day 8 of illness, HLH work-up was initiated. Serum ferritin was elevated (11167 ng/mL) with hypofibrinogenemia (152.3 mg/dL) and hypertriglyceridemia (279mg/dL). Brain natriuretic peptide was very high (6622 pg/mL) and echocardiogram (ECHO) revealed left ventricular dysfunction (ejection fraction-45%, normal being >65%) suggesting myocarditis. Hence, she was started on dobutamine and milrinone infusion. Bone marrow aspiration showed hemophagocytes (Figure.1). Blood culture grew Salmonella typhi, which was sensitive to ceftriaxone. In view of worsening clinical condition and elevated inflammatory markers, child was given intravenous immunoglobulin 2 g/kg over 48 hrs. Child gradually improved, fever spikes started decreasing, tachypnea settled and she was weaned off from HFNC and inotropic support. She was continued on

Figure 1 Leishman Stain 100X magnification. Arrow pointing to a hemophagocyte



enalapril, aspirin, furosemide and spironolactone. On follow up at 4 weeks, ECHO was normal and anti-failure measures were stopped.

Case 2

A 4-year-old girl presented with fever and skin rash over the lower limbs that commenced 5 and 3 days ago, respectively. She also had vomiting and reduced urine output for a day. On examination, she was conscious and oriented. Her heart rate was 170/min, respiratory rate was 40/min, blood pressure was 70/50 mmHg with cool peripheries, feeble peripheral pulses and capillary refill time > 3 seconds. She had macular erythematous blanching rash over both lower limbs and forearms. Her liver was enlarged to 4 cm, soft in consistency, with mild tenderness. Other examinations were normal.

Management and outcome

She was provisionally diagnosed to have dengue fever with hypotensive shock and resuscitated with 30 ml/kg normal saline fluid bolus. She had severe thrombocytopenia (30 $\times 10^9$/L) with hemoconcentration (Hb- 14.5 g/dL; PCV 48). Her liver enzymes were elevated (SGOT: 524 U/L and SGPT: 124 U/L). Dengue IgM was positive. On day 3 of admission, she had persistent fever and developed pleural effusion, requiring oxygen support. In view of persistent fever for 8 days, HLH workup was done. Child had hyperferritinemia (7500 ng/mL), hypofibrinogenemia

(132.7 mg/dL) and hypertriglyceridemia (251 mg/dL). ECHO was normal. Intravenous immunoglobulin was given at 2 g/kg over 48 hours. During the next 48 hours, her fever spikes settled and platelet count started increasing and she was discharged.

Laboratory parameters of both cases have been listed in Table 1.

DISCUSSION

Hemophagocytic lymphohistiocytosis is an immune-mediated life-threatening hyperinflammatory syndrome, which was initially described by pediatricians Robb-Smith and Scott in 1939. It is characterized by uninterrupted hyperinflammatory response associated with abnormal activation of macrophages and lymphocytes, resulting in hypercytokinemia (3)(4). Primary HLH occurs due to a variety of genetic abnormalities and frequently presents during infancy and early childhood, while secondary HLH is less age-restricted and it is more common in older children and adults. HLH can be triggered by a variety of events that disrupt immune homeostasis, with infection being a common trigger in both genetic and sporadic cases. Viruses are most commonly associated with secondary HLH, particularly Epstein-Barr virus, but tuberculosis, malaria, dengue, leishmaniasis and typhoid are important infections that trigger HLH, especially in tropical countries (5). In the present report, the triggers implicated were dengue and typhoid fever.

The pathogenetic mechanisms of HLH is impaired activation of innate immune system, precisely natural killer (NK) cells and CD 8+ cytotoxic T-lymphocytes (CTL), resulting in release of abundant inflammatory cytokines that promote cytokine network formation and macrophage infiltration. The liver, spleen and lungs are the commonly affected organs, but HLH can affect all organs of the body. In a healthy immune system, CD 8+ cytotoxic T-lymphocytes and NK cells secrete two cytolytic enzymes: granzyme and

Table 1 Laboratory values of the cases

Parameter	Case 1	Case 2	Normal values
Total Count (cells/mm^3)	3820	5700	5000 - 17000
Hb (g/dL)	8.4	10.8	11-14
Platelets (×10^9/L)	36	30	150-400
SGOT (U/L)	368	524	<35
SGPT (U/L)	167	124	13-45
Ferritin (ng/mL)	11167	7500	13 - 150
Triglyceride (mg/dL)	279	251	<150
Fibrinogen (mg/dL)	152.3	132.7	170 - 405

perforin. Perforin creates destabilizing pores in the membrane of the target cell which results in target cell destruction.

In patients with HLH, dysfunction of these cytosolic proteins causes reduced function of CD 8+ cytotoxic T-lymphocytes and NK-cell. The impaired cells loose its ability to eliminate virus-infected cells and instead uninterruptedly secrete inflammatory cytokines. In addition, these cytokines activate antigen-presenting cells (histiocytes and macrophages) to produce more cytokines. This results in a vicious cycle that amplifies the cytokine secretion of CD 8+ T-lymphocytes, NK cells, and macrophages, thus generating a cytokine storm. Hyperproduction of cytokines, including tumour necrosis factor α, interleukin 6 and interferon γ by virus-infected T-lymphocytes may play a role in the pathogenesis of dengue-associated HLH. Recently, enhanced antigen presentation and repetitive interferon γ-dependent stimulation of Toll-like receptors (TLRs) have also been postulated as causal mechanisms of reactive HLH. Direct activation of TLRs by intracellular pathogens that persist in histiocytes may explain the pathogenesis of typhoid-associated HLH.

The clinical features of HLH can be explained by 3 cellular pathways: 1) uncontrolled activation of macrophages and CD8+ T-lymphocytes; 2) hyperproliferation and infiltration of these cells into various organs; and 3) hypercytokinemia, resulting in progressive multi-organ dysfunction. Early symptoms of HLH are non-specific and can mimic common infections. The cardinal symptoms of the disease include continuous fever (>38·5°C), enlarged lymphohemopoietic organs and bi- or tri-lineage cytopenia. Neurological abnormalities are observed in nearly one-third of patients with HLH. The disease can also affect other vital organ systems, including the respiratory system, heart, kidneys and skin. Both the children presented here had lung involvement and one child had myocarditis in addition to respiratory distress.

Diagnosis of secondary HLH is challenging because of nonspecific clinical features. Hemophagocytic lymphohistiocytosis should be clinically suspected in all patients presenting with undiagnosed, continuous high-grade fever and evidence of multi-organ involvement. The diagnostic criteria proposed by the HLH-2004 study, requires 5 out of 8 parameters for the diagnosis of HLH (Table 2)(6). An elevated serum ferritin level is very common in children with HLH and has high sensitivity and specificity. Most patients with HLH will have hepatitis, characterised by elevated liver enzymes, lactate dehydrogenase and bilirubin. Hypertriglyceridemia and abnormal coagulation parameters are also frequently seen. The detection of hemophagocytosis, a hallmark of activated macrophages in the bone marrow, is supportive for diagnosis, however bone marrow biopsies are neither specific nor sensitive for conclusive diagnosis. Recently, flow cytometry has been implicated as a screening tool for identifying patients with genetic predisposition to HLH. Transient defect in NK cells and CTL cells function may be noted in secondary HLH while the changes will be persistent in primary HLH (7). Similar manifestation of continuous fever with multi-organ involvement can happen in multisystem inflammatory syndrome in children (MIS-C) following COVID-19 infection. But both the patients were negative for COVID-19 serology.

The initial goal of therapy in patients with HLH is to suppress the unregulated severe hyperinflammation, since both genetic and acquired forms of HLH can initially be managed by the same treatment protocol. Following this, the goal of therapy shifts towards identification of underlying triggering agents, as patients with primary HLH may require hematopoietic stem cell transplantation. Most cases of secondary

HLH resolve once the underlying disease is treated like in our children.

Regarding treatment of dengue-associated HLH, other similar cases reported in the literature showed that few cases have recovered with supportive therapy only. Intravenous immunoglobulin G has been used in few cases either alone or with steroids (8)(9). The treatment of dengue-associated HLH using intravenous immunoglobulin G seems to be associated with favourable outcome, as in this case report. On the other hand, typhoid is one of the bacterial infections, that can be a potential trigger for HLH. Treatment of the inciting bacterial pathogen is often inadequate and in most of the cases it is essential to start lifesaving therapy with immunosuppressants and immunomodulators as early as possible to prevent mortality(10).

Given the poor outcomes of HLH and the rapidity with which the disease progresses to cause fatal multi-organ failure, it is essential that signs and symptoms of hypercytokinemia are not overlooked and that the hyperinflammatory state is suppressed as soon as possible. In addition, it is imperative to detect the triggering event in secondary HLH and initiate prompt treatment to achieve successful outcome.

❖❖❖❖❖

Compliance with ethical standards

- **Conflict of interests**: The authors have declared that no Conflict of interest exists.
- **Ethical approval**: It was not obtained as this is a case report.
- **Informed consent**: Informed consent was obtained from the participants parents.
- **Funding**: The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Table 2 Diagnostic criteria for hemophagocytic lymphohistiocytosis based on HLH-2004

Diagnostic criteria for hemophagocytic lymphohistiocytosis (HLH)
1. Persistent fever - >/=38.5°C
2. Splenomegaly
3. Bicytopenia or pancytopenia (Hb<9 g%, Platelets<1 lakh/µL, Neutophils<1000/µL)
4. Hypertriglyceridemia and/or hypofibrinogenemia
5. Low or absent NK cell activity
6. Hyperferritenemia (>500 ng/mL)
7. High sCD25 (sIL-2R>2,400 U/ml)
8. Hemophagocytosis in bone marrow, spleen, lymph nodes or liver

❖❖❖❖❖

LEARNING POINTS

- Secondary hemophagocytic lymphohistiocytosis should be suspected in all children presenting with continuous high-grade fever beyond the natural course of illness and evidence of multi-organ involvement.
- Prompt treatment of the underlying triggering event of secondary HLH is essential
- It is prudent to start lifesaving therapy with immunosuppressants and immunomodulators in case of hypercytokinemia leading to multi organ involvement.

REFERENCES

1. Ishii E. Hemophagocytic Lymphohistiocytosis in Children: Pathogenesis and Treatment. Front Pediatr. 2016; 4:47.

2. Janka GE, Lehmberg K. Hemophagocytic syndromes--an update. Blood Rev. 2014 Jul;28(4):135–42.

3. Al-Samkari H, Berliner N. Hemophagocytic Lymphohistiocytosis. Annu Rev Pathol. 2018 Jan;13:27–49.

4. Canna SW, Marsh RA. Pediatric hemophagocytic lymphohistiocytosis. Blood. 2020 Apr;135(16):1332–43.

5. Fisman DN. Hemophagocytic syndromes and infection. Emerg Infect Dis. 2000;6(6):601–8.

6. Henter J-I, Horne A, Aricó M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer. 2007 Feb;48(2):124–31.

7. Madkaikar MR, Shabrish S, Kulkarni M, Aluri J, Dalvi A, Kelkar M, et al. Application of Flow Cytometry in Primary Immunodeficiencies: Experience From India. Front Immunol. 2019;10:1248.

8. Thadchanamoorthy V, Dayasiri K. Dengue Fever Associated Haemophagocytic Lymphohistiocytosis: A Report of Two Children. Vol. 12, Cureus. 2020. p. e11232.

9. Pal P, Giri PP, Ramanan A V. Dengue associated hemophagocytic lymphohistiocytosis: a case series. Indian Pediatr. 2014 Jun;51(6):496–7.

10. Uribe-Londono J, Castano-Jaramillo LM, Penagos-Tascon L, Restrepo-Gouzy A, Escobar-Gonzalez A-F. Hemophagocytic Lymphohistiocytosis Associated with Salmonella typhi Infection in a Child: A Case Report with Review of Literature. Classen C, editor. Case Rep Pediatr [Internet]. 2018;2018:6236270. Available from: https://doi.org/10.1155/2018/6236270.

Editor-in-chief

János Kappelmayer
Department of Laboratory Medicine
University of Debrecen, Hungary

Assistant Editor

Harjit Pal Bhattoa
Department of Laboratory Medicine
University of Debrecen, Hungary

Editorial Board

Khosrow Adeli, The Hospital for Sick Children, University of Toronto, Canada

Borut Božič, University Medical Center, Lubljana, Slovenia

Edgard Delvin, CHU Sainte-Justine Research Center, Montréal, Québec, Canada

Nilda E. Fink, Universidad Nacional de La Plata, Argentina

Ronda Greaves, Biochemical Genetics, Victorian Clinical Genetics Services, Victoria, Australia

Mike Hallworth, Shrewsbury, United Kingdom

Andrea R. Horvath, Prince of Wales Hospital and School of Medical Sciences, University of New South Wales, Sydney, Australia

Ellis Jacobs, EJ Clinical Consulting, LLC, USA

Allan S. Jaffe, Mayo Clinic, Rochester, USA

Bruce Jordan, Roche Diagnostics, Rotkreuz, Switzerland

Gábor L. Kovács, University of Pécs, Hungary

Evelyn Koay, National University, Singapore

Tamas Kőszegi, University of Pécs, Hungary

Janja Marc, University of Ljubljana, Slovenia

Gary Myers, Joint Committee for Traceability in Laboratory Medicine, USA

Tomris Ozben, Akdeniz University, Antalya, Turkey

Maria D. Pasic, Laboratory Medicine and Pathobiology, University of Toronto, Canada

Maria del C. Pasquel Carrera, College of Chemists, Biochemists and Pharmacists, Pichincha, Ecuador

Oliver Racz, University of Kosice, Slovakia

Rosa Sierra Amor, Laboratorio Laquims, Veracruz, Mexico

Sanja Stankovic, Institute of Medical Biochemistry, Clinical Center of Serbia, Belgrade, Serbia

Danyal Syed, Ryancenter, New York, USA

Grazyna Sypniewska, Collegium Medicum, NC University, Bydgoszcz, Poland

Peter Vervaart, LabMed Consulting, Australia

Stacy E. Walz, Arkansas State University, USA

Publisher: IFCC Communications and Publications Division (IFCC-CPD)

Copyright © 2022 IFCC. All rights reserved.

The eJIFCC is a member of the **Committee on Publication Ethics (COPE).**

The eJIFCC (Journal of the International Federation of Clinical Chemistry) is an electronic journal with frequent updates on its home page. Our articles, debates, reviews and editorials are addressed to clinical laboratorians. Besides offering original scientific thought in our featured columns, we provide pointers to quality resources on the World Wide Web.

This is a Platinum Open Access Journal distributed under the terms of the *Creative Commons Attribution Non-Commercial License* which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Produced by: Insoft Digital Web Solutions

Published by: IFCC International Federation of Clinical Chemistry and Laboratory Medicine

www.ifcc.org