ACTA ABP BIOCHIMICA POLONICA

Vol. 70, No 4/2023
885–889
https://doi.org/10.18388/abp.2020_6729

Regular paper

Mutational analysis of *FOLR1* and *FOLR2* genes in children with Myelomeningocele

Nageen Hussain[1], Saira Malik[1], Tayyaba Faiz[1], Fiza Shafqat[1], Ayaz Ali Khan[2], Taqweem Ul Haq[2], Waqar Ali[2], Tariq Aziz[3] ✉, Metab Alharbi[4], Abdulrahman Alsahammari[4] and Abdullah F. Alasmari[4]

[1]Institute of Microbiology and Molecular Genetics, New Campus, University of the Punjab, Lahore, Pakistan; [2]Department of Biotechnology University of Malakand 18800 Pakistan; [3]Department of Agriculture, University of Ioannina, 47100 Arta, Greece; [4]Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

Myelomeningocele (MMC) is a congenital disease. For a long time, molecular mechanism of MMC, the role of folate receptor and transporter proteins remain unclear. Folate from maternal lumen to developing embryo is carried out with the help of folate transporters (SLC46A1, SLC19A1, FOLH1 and SLC25A32) and folate receptor (FOLR1, FOLR2 and FOLR3). Due to the loss of function of these important genes, complications can facilitate the risk of MMC. This study focused on the mutational analysis of *FOLR1* and *FOLR2* genes in children suffering from MMC. Myelomeningocele is a rare disorder so twenty blood samples from the children were collected. Primers of selected exons for *FOLR1* and *FOLR2* genes were designed with the help of PrimerFox software. Extracted DNA was amplified, and PCR based mutational analysis was done to check any type of mutation/SNPs in these genes. Sanger sequencing method was performed to confirm mutation in *FOLR1* and *FOLR2* genes. The results showed that certain environmental factors (smoking, low socio-economic status of mother bearing MMC fetus) were found to be significantly ($P<0.05$) associated with MMC but no mutation in the selected exons of *FOLR1* and *FOLR2* genes was detected. Thus, genetic variations in the folate transporter gene may have no role in the progression of MMC in the studied population.

Keywords: FOLR1, FOLR2, Congenital, Myelomeningocele, phenotype, mutation

Received: 10 March, 2023; revised: 08 August, 2023; accepted: 25 August, 2023; available on-line: 26 October, 2023

✉e-mail: iwockd@gmail.com
Acknowledgments of Financial Support: The authors greatly acknowledge and express their gratitude to the Researchers Supporting Project number (RSP2023R335), King Saud University, Riyadh, Saudi Arabia
Abbreviations: CSF, Cerebrospinal fluid; FOLR1, Folate Receptor Alpha; FOLR2, Folate Receptor Beta; MMC, Myelomeningocele

INTRODUCTION

One of the most important and congenital forms of spina bifida is Myelomeningocele (MMC). In this disease, two sides of backbone become unable to fuse with one another and forms a gap between them (Hassan *et al.,* 2022). This usually happens after about 2–4 weeks of gestation. Due to the failure of fusion of both backbone side nerves, spinal cord and meninges remain uncovered. This can lead to the partial or complete paralysis of the body. Partial paralysis happens in the lower portion of the body from the point of opening (Moldenhauer & Adzick, 2017). The evidence of this disease in first-degree relatives and second-degree relatives is about 3–4% and 1–2%, respectively (Farmer *et al.,* 2018). Genomic and environmental factors are responsible for this situation. Folic acid plays a vital role in the organ development of fetus in placenta (O'Byrne *et al.,* 2010). Genes involved in folate transport play an important contribution in the development of MMC. Other environmental factors such as deficiency of vitamins, certain medications, tobacco usage, drug addiction, maternal infections, maternal hyperthermia and exposure to some metals play vital role in tube defects in developing fetus (Naveed *et al.,* 2023; Muhammad *et al.,* 2023; Naveed *et al.,* 2022; Tauheed *et al.,* 2017).

Studies on animal models illustrate involvement of about 200 genes in tube defects (Cavalheiro *et al.,* 2021). It is proposed that certain genes and their variants make an interaction with each other and, being affected by environmental factors, cause MMC (Mazumdar *et al.,* 2015). Certain studies highlighted certain mutations and/or SNPs i.e. rs223622 and rs180113 in the MTHFR gene, MTHFDM1 gene, rs13908 in FOLR2 and rs792687, rs792554 and rs792698 in FOLR3 gene (Ntimbani *et al.,* 2020). These mutations have been reported in different ethnic groups, such as Dutch, Italian, and British population. The role of folate transporter genes has already been documented worldwide (Wolujewicz *et al.,* 2021). Unfortunately, little work has been done on this among the Pakistani population so far. The main objective of this study is to check the role of environmental factors and to perform the mutational analysis of *FOLR1* and *FOLR2* genes with reference to Myelomeningocele.

METHODOLOGY

All the patients in this study had less than 2 years of age. Before the procedure, informed consent of the parents was taken. The skin of the patients was sterilized with alcohol swabs. About 2 to 3cc blood of MMC patients was collected with the help of sterilized syringes and stored in refrigerator at 4°C in an EDTA vacutainer. The sample of mothers of MMC patients was also collected when it was possible. DNA extraction was done by using the standard method (Albertsen *et al.,* 2015). Forward and reverse primers were prepared for exons 3–4 and 5 for *FOLR1* gene, and exons 3–4, 5 of *FOLR2* gene. Primers were designed by using PrimerFox Software (Table 1).

Table 1. Forward and reverse primers for exons 3–4 and 5 of *FOLR1* and *FOLR2* genes

Primers	Sequence (5′-3′)	Tm (ºC)	Length (nt)
FOLR2 exon 5 forward	TCCTGGATGGCCCTTATG	57	19
FOLR2 exon 5 reverse	GCAACAGATGGGTGACAGA	59	20
FOLR2 exon 3–4 forward	ACCATCACTGGGAACCTGA	58	20
FOLR2 exon 3–4 reverse	CAGCTGGCACTTGTTAACTC	61	20
FOLR1 Exon 5 forward	ATTTGGAGTTGTAGGGCTG	55.2	19
FOLR1 Exon 5 reverse	TTCTCAAGACACATGTGCG	56.7	
FOLR1 Exon 3–4 forward	GCTGGGGAATCAAGGACTA	59	19
FOLR1 Exon 3–4 reverse	GCCCGGAACTATCTTGAGGT	59	

Table 2. Association of Environmental factors with MMC

Characteristics	MMC (n=20)	Controls (n=20)	OR (95%CI)	*P*-value
Smoking				
Exposed	14.0 (70)	7.0 (35)	4.33 (1.115–16.32)	0.0302
Not exposed	6.0 (30)	13.0 (65)		
Supplements (Folic acid) intake				
Yes	10.0 (50%)	8.0 (40%)	1.5 (0.42–5.24)	0.52
No	10.0 (50%)	12.0 (60%)		
Weight of Mother				
Under	16.0 (80%)	12.0 (60%)	2.66 (0.64–10.9)	0.17
Over	4.0 (20%)	8.0 (40%)		
History of Abortions/Premature birth				
Yes	7.0 (35%)	5.0 (25%)	1.615 (0.41–6.33)	0.491
No	13.0 (65%)	15.0 (75%)		
Age of Mother (Years)				
Under 30	14.0 (70%)	17.0 (85%)	0.411 (0.086–1.95)	0.263
Above 30	6.0 (30%)	3.0 (15%)		
Family income				
Poor	12.0 (60%)	4.0 (20%)	6 (1.45–24.6)	0.013
Middle + affluent	8.0 (40%)	16.0 (80%)		

The standard PCR procedure was followed for the amplification of *FOLR1* and *FOLR2* genes (Lorenz, 2012). The denaturation temperature was 95°C for both *FOLR1* and *FOLR2* genes and it was done for 4 minutes. The annealing temperature for *FOLR1* gene was 58°C for 45 seconds and 51–59°C for *FOLR2* gene and it was done for about 45 seconds. Elongation temperature was 72°C and it was done for one minute and finally for 4 minutes. To get the appropriate amount of PCR product, 35 cycles of PCR were run. Holding temperature of the PCR vials was 4°C. Afterward, the PCR product was loaded on agarose gel and visualized with the help of UV light. To rule out mutation sequencing of the amplified products Sanger Sequencing method was done. Statistical analysis was done with the use of GraphPad Prism software v13.

RESULTS

Current study includes 20 MMC patients. Most of the patients lie within the age group of (1–2 years). Patients usually belong to Northern and Southern Punjab and most of the mothers were malnutritional due to poverty as shown in Fig. 1. The statistical analysis was done to check the association of different environmental factors (Smoking, weight/age of the mother, Folic acid intake, history of abortion and family socio-economic status) with MMC. Smoking and poor socio-economic status of

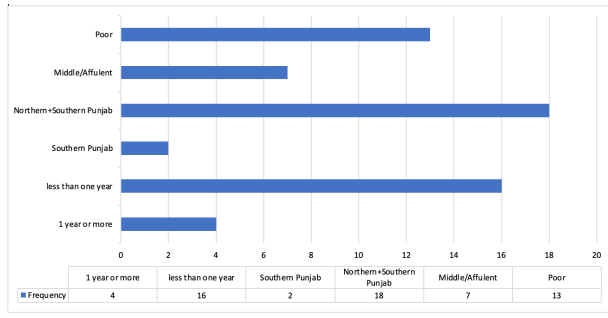

Figure 1. Age groups, geographical location and economic background of subjects

Table 3. Location of MMC and hydrocephalus common in the patients (n=20)

		Frequency
Location		
Valid	Lumber	12 (60%)
	Cervical	7 (35%)
	Both	1 (5%)
	Total	20 (100%)
Hydrocephalus		
Valid	No	14 (70%)
	Yes	6 (30%)
	Total	20 (100%)

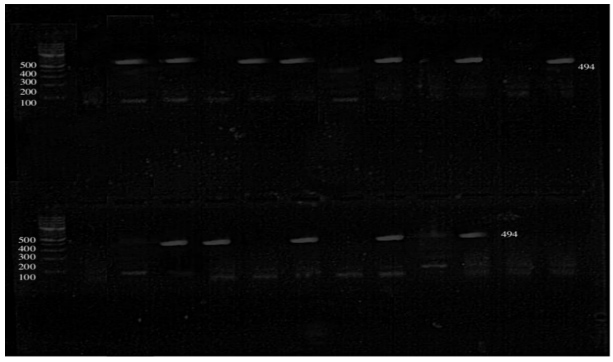

Figure 2. Amplified DNA band of Exon 5 *FOLR1* gene in patients

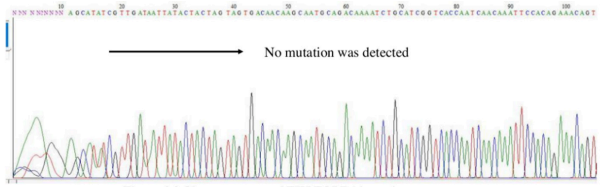

Figure 3. Chromatogram of Exon 5 *FOLR1* gene in MMC patients

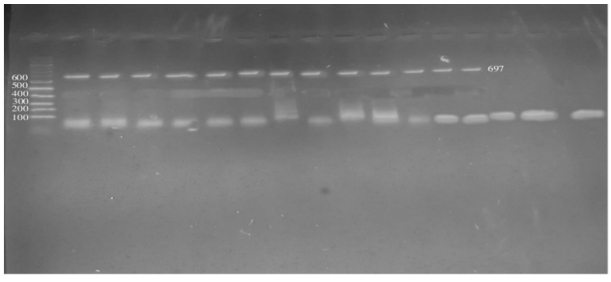

Figure 4. Amplified DNA Band of exon 3–4 of *FOLR1* gene in patients.

the mother was found to be significantly associated with MMC as the *P*-value is less than 0.05 (Table 2).

If we look at the clinical symptoms, 70% (n=14) of the patients were hydrocephalus. The position of Myelomeningocele varies among patients, 35% (n=7) had in cervical region, 60% (n=12) covered lumbar region while 5% (n=1) showed in both cervical and lumbar regions (Table 3).

Primer was designed for exon 5 of *FOLR1* gene. After performing PCR, PCR product was visualized on 1.5% agarose gel. There was a clear bright band on 494bp location which indicates no mutation in it (Fig. 2). Further, sequencing was done, and the chromatogram also showed no mutations (Fig. 3).

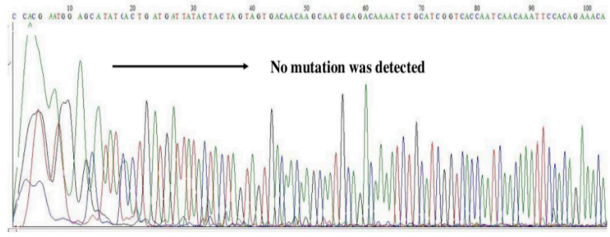

Figure 5. Chromatogram of Exon 3–4 *FOLR1* in patients

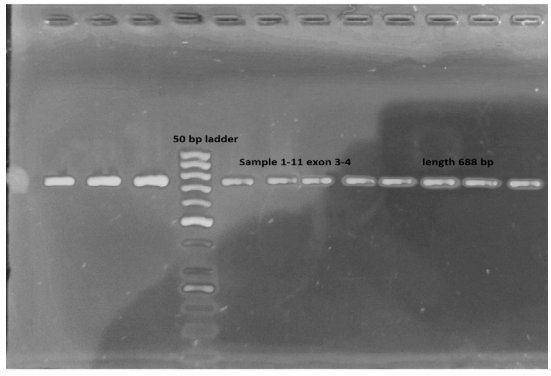

Figure 6. Amplified DNA Band of exon 3–4 of *FOLR2* gene in patients

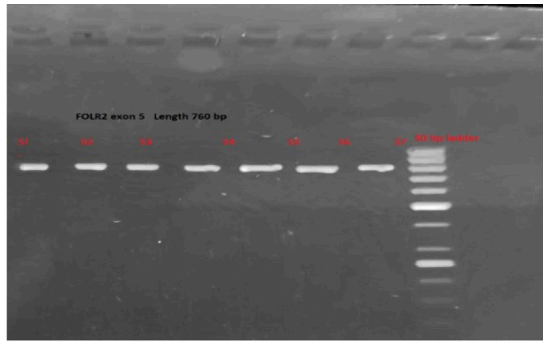

Figure 7. Amplified DNA Band of exon 5 of *FOLR2* gene in patients

Exons 3-4 of *FOLR1* gene primers were also designed and the band of interest was of 679bp. This clearly indicates no mutation in these exons as well (Fig. 4). This was also confirmed by the chromatogram of these exons in *FOLR1* gene (Fig. 5).

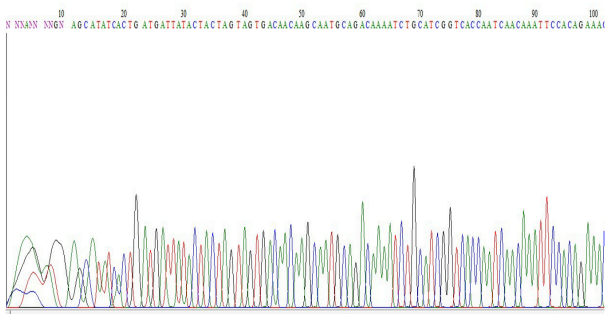

Figure 8. Chromatogram showing exons 3–4 sequences of *FOLR2* gene.

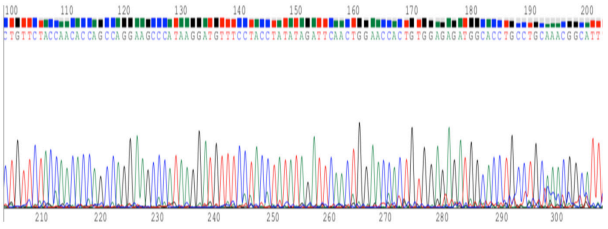

Figure 9. Chromatogram showing exon 5 sequences of *FOLR2* gene.

FOLR2 is another important gene involved in MMC. So, primers were designed for exons 3–4, 5 and PCR was performed. The PCR product for exons 3–4 showed a clear band of 686bp (Fig. 6) and exon 5 of 760 bp (Fig. 7) which indicates that there is no mutation in these exons as well and it was confirmed by sequencing (Figs 8 and 9).

DISCUSSION

Myelomeningocele is an important congenital disorder. It usually occurs after the 2–4 weeks of pregnancy. Folic acid deficiency can lead towards the exaggeration of MMC disease. Folate is needed for fetal organ development (Mazumdar *et al.,* 2015). Its demand increases significantly during pregnancy and is found to be significantly associated with MMC. Due to pediatric neurodegeneration resulting from neural folate deficiency, the role of FR can significantly inhibit folate uptake in the CSF (Canfield *et al.,* 2009). In the present study, various environmental factors like folic acid intake, body weight and age of mother, and history of abortion were found to be insignificantly associated with MMC ($P>0.05$). However smoking habit in mother in the form of hukkah, cigarettes, sheesha and burning of fossil fuels was found to be significantly associated with MMC. Similarly, a poor family socio-economic status was found to be significantly associated with MMC ($P<0.05$). (Nageen *et al.,* 2023; Hussain *et al.,* 2023).

Defects in folic acid receptor or transport protein can lead towards the MMC disorder. Many studies have been done for the mutational analysis of *FOLR1* and *FOLR2* genes but only a few studies have reported some mutations in these genes. Studies done in 2014 and 2020 indicated some novel mutations in these genes. (Tenpenny *et al.,* 2014; Steele *et al.,* 2020). In 2017, a study was done in the United States of America and it showed the down regulation of *FOLR1* gene in MMC patients (Findley *et al.,* 2017). In 2020, a study confirmed twelve new mutations in Folate receptor genes (Hillman *et al.,* 2020). This

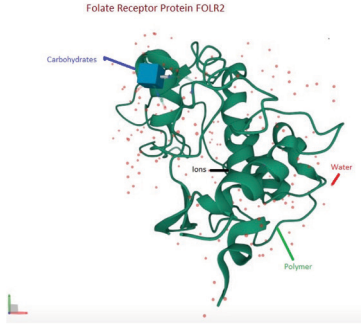

Figure 10. FOLR2 protein structure in MMC patients (n=20)

proved the direct link of FOLR1 protein in the disease. Just like Boyles *et al.,* the present study also showed no mutation in *FOLR1* and *FOLR2* genes (Boyles *et al.,* 2006).

In 2022, another study showed the low level of FOLR1 protein (known as glycosyl-phosphatidylinositol-anchored plasma membrane protein) in MMC patients as compared to healthy people. *FOLR2* gene is present on chromosome 11 and belongs to the folate receptor family, producing folate receptor beta protein. FRβ, is a member of this family of reduced folate (Han *et al.,* 2022). In the present study, MMC patients showed no alteration in the cluster of Folate receptor protein FOLR2 (Fig. 10).

CONCLUSION

This study showed no mutation in *FOLR1* gene exons 3–4 and 5, and *FOLR2* gene exons 3-4 and 5. This study emphasises the significance of maternal health and dietary consumption in determining the trajectory of these receptor genes, especially FOLR1 and FOLR2, given the critical role of folate in satisfying the developmental demands of a growing fetus. The results of this study indicate that genetic variations in the folate receptor genes may not significantly contribute to the development of MMC (Myelomeningocele), but environmental factors are more important in this situation. The limitation of this study includes small sample size, as it is a rare disease. Furthermore, this study lacks ultrasound report of the infants or toddlers, maternal folic acid levels and other blood profile data during and before conceiving.

Declarations

Author Contributions. Conceptualization, N.H, S.M, T.F, F.S and T.A.; methodology, N.H, S.M, T.F, F.S; software, T.A; validation, A.A.S; formal analysis, T.A.; investigation, N.H, S.M, T.F, F.S; resources, M.A and A.A.S.; data curation, T.A.; writing – original draft preparation, T.A and E.A; writing – review and editing, A.A. K, T.Q, WA and A.F.A; visualization, A.A.S; supervision, T.A and B.I.; project administration, A.A.S and M.A; funding acquisition, T.A.

Funding. This research work received no external funding.

Conflict of interest. The authors have declared no conflict of interest.

REFERENCES

Albertsen M, Karst SM, Ziegler AS, Kirkegaard RH, Nielsen PH (2015) Back to basics – The influence of DNA extraction and

primer choice on phylogenetic analysis of activated sludge communities. *PLoS One* **10**: e0132783. https://doi.org/10.1371/journal.pone.0132783

Boyles AL, Billups AV, Deak KL, Siegel DG, Mehltretter L, Slifer SH, Bassuk AG, Kessler JA, Reed MC, Nijhout HF, George TM, Enterline DS, Gilbert JR, Speer MC NTD Collaborative Group (2006) Neural tube defects and folate pathway genes: family-based association tests of gene–gene and gene-environment interactions. *Environ Health Perspect* **114**: 1547–1552. https://doi.org/10.1289/ehp.9166

Canfield MA, Marengo L, Ramadhani TA, Suarez L, Brender JD, Scheuerle A (2009) The prevalence and predictors of anencephaly and spina bifida in Texas. *Paediatr Perinat Epidemiol* **23**: 41–50. https://doi.org/10.1111/j.1365-3016.2008.00975.x

Cavalheiro S, Costa MDS, Barbosa MM, Patricia AD, Jardel NM, Daniela C, Antonio FM (2021) Hydrocephalus in myelomeningocele. *Childs Nerv Syst* **37**: 3407–3415. https://doi.org/10.1007/s00381-021-05333-2

Farmer DL, Thom EA, Brock JW 3rd, Burrows PK, Johnson MP, Howell LJ, Farrell JA, Gupta N, Adzick NS (2018) Management of Myelomeningocele Study Investigators. The Management of Myelomeningocele Study: full cohort 30-month pediatric outcomes. *Am J Obstet Gynecol* **218**: 256.e1–256.e13. https://doi.org/10.1016/j.ajog.2017.12.001

Findley TO, Tenpenny JC, O'Byrne MR, Morrison AC, Hixson JE, Northrup H, Au KS (2017) Mutations in folate transporter genes and risk for human myelomeningocele. *Am J Med Genet A* **173**: 2973–2984. https://doi.org/10.1002/ajmg.a.38472

Han X, Cao X, Aguiar-Pulido V, Yang W, Karki M, Ramirez PAP, Cabrera RM, Lin YL, Wlodarczyk BJ, Shaw GM, Ross ME, Zhang C, Finnell RH, Lei Y (2022) CIC missense variants contribute to susceptibility for spina bifida. *Hum Mutat* **43**: 2021–2032. https://doi.org/10.1002/humu.24460

Hassan AS, Du YL, Lee SY, Wang A, Farmer DL (2022) Spina bifida: a review of the genetics, pathophysiology and emerging cellular therapies. *J Dev Biol* **10**: 22. https://doi.org/10.3390/jdb10020022

Hillman P, Baker C, Hebert L, Brown M, Hixson J, Ashley-Koch A, Morrison AC, Northrup H, Au KS (2020) Identification of novel candidate risk genes for myelomeningocele within the glucose homeostasis/oxidative stress and folate/one-carbon metabolism networks. *Mol Genet Genomic Med* **8**: e1495. https://doi.org/10.1002/mgg3.1495

Hussain N, Mumtaz M, Adil M, Ali Nadeem A, Sarwar A, Aziz T, Alharbi M, Alshammari A, Alasmari AF, Alharbi ME (2023) Investigation of VEGF (rs 699947) polymorphism in the progression of Rheumatoid Arthritis (RA) and *in-silico* nanoparticle drug delivery of potential phytochemicals to cure RA. *Acta Biochim Pol* **70**: 591–598. https://doi.org/10.18388/abp.2020_6654

Lorenz TC (2012) Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *J Vis Exp* **22**: e3998. https://doi.org/10.3791/3998

Mazumdar M, Ibne Hasan MO, Hamid R, Valeri L, Paul L, Selhub J, Rodrigues EG, Silva F, Mia S, Mostofa MG, Quamruzzaman Q, Rahman M, Christiani DC (2015) Arsenic is associated with reduced effect of folic acid in myelomeningocele prevention: a case control study in Bangladesh. *Environ Health* **14**: 34. https://doi.org/10.1186/s12940-015-0020-0

Mazumdar M, Valeri L, Rodrigues EG, Ibne Hasan MO, Hamid R, Paul L, Selhub J, Silva F, Mostofa MG, Quamruzzaman Q, Rahman M, Christiani DC (2015) Polymorphisms in maternal folate pathway genes interact with arsenic in drinking water to influence risk of myelomeningocele. *Birth Defects Res A Clin Mol Teratol* **103**: 754–762. https://doi.org/10.1002/bdra.23399

Moldenhauer JS, Adzick NS (2017) Fetal surgery for myelomeningocele: After the Management of Myelomeningocele Study (MOMS) *Semin Fetal Neonatal Med* **22**: 360–366. https://doi.org/10.1016/j.siny.2017.08.004

Muhammad N, Ain, T Aziz, I Ali, M Aqib Shabbir, K Javed, M Alharbi, A Alshammari, AF Alasmari (2023) Revolutionizing treatment for toxic shock syndrome with engineered super chromones to combat antibiotic-resistant *Staphylococcus aureus*. *Eur Rev Med Pharmacol Sci* **27**: 5301–5309. https://doi.org/10.26355/eurrev_202306_32649

Nageen H, Alkhateeb MA, Bashir A, Iqbal R, Hussain T, Aziz T, AlAbbas NS, Shaer NA, Alamri AS, Alhomrani M, Shakoori A, Labban S (2023) Deletions of SMNI gene exon 7 and NAIP gene exon 5 in spinal muscular atrophy patients in selected population. *Eur Rev Med Pharmacol Sci* **27**: 7935–7945. https://doi.org/10.26355/eurrev_202309_33552

Naveed M, Ali U, Aziz T, Rasool MJ, Ijaz A, Alharbi M, Alharbi ME, Alshammari A, Alasmari AF (2023) A reverse vaccinology approach to design an mRNA-based vaccine to provoke a robust immune response against HIV-1. *Acta Biochim Pol* **70**: 407–418. https://doi.org/10.18388/abp.2020_6696

Naveed M, Sheraz M, Amin A, Waseem M, Aziz T, Khan AA, Ghani M, Shahzad M, Alruways MW, Dablool AS, Ahmad ME, Abdulhakeem AA,Abdulhakeem SA, Majid A (2022) Designing a novel peptide-based multi-epitope vaccine to evoke a robust immune response against pathogenic multidrug-resistant providencia heimbachae. *Vaccines* **10**: 1300. https://doi.org/10.3390/vaccines10081300

Ntimbani J, Kelly A, Lekgwara P (2020) Myelomeningocele-A literature review. *Interdiscip Neurosurg* **19**: 100502. https://doi.org/10.1016/j.inat.2019.100502

O'Byrne MR, Au KS, Morrison AC, Lin JI, Fletcher JM, Ostermaier KK, Tyerman GH, Doebel S, Northrup H (2010) Association of folate receptor (FOLR1, FOLR2, FOLR3) and reduced folate carrier (SLC19A1) genes with meningomyelocele. *Birth Defects Res A Clin Mol Teratol* **88**: 689–694. https://doi.org/10.1002/bdra.20706

Steele JW, Kim SE, Finnell RH (2020) One-carbon metabolism and folate transporter genes: Do they factor prominently in the genetic etiology of neural tube defects? *Biochimie* **173**: 27–32. https://doi.org/10.1016/j.biochi.2020.02.005

Tauheed J, Sanchez-Guerra M, Lee JJ, Paul L, Ibne Hasan MOS, Quamruzzaman Q, Selhub J, Wright RO, Christiani DC, Coull BA, Baccarelli AA, Mazumdar M (2017) Associations between post translational histone modifications, myelomeningocele risk, environmental arsenic exposure, and folate deficiency among participants in a case control study in Bangladesh. *Epigenetics* **12**: 484–491. https://doi.org/10.1080/15592294.2017.1312238

Wolujewicz P, Steele JW, Kaltschmidt JA, Finnell RH, Ross ME (2021) Unraveling the complex genetics of neural tube defects: From biological models to human genomics and back. *Genesis* **59**: e23459. https://doi.org/10.1002/dvg.23459.