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POPULATION STUDY ARTICLE

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Bronchopulmonary dysplasia and wnt pathway-associated single nucleotide polymorphisms

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AIM: Genetic variants contribute to the pathogenesis of bronchopulmonary dysplasia (BPD). The aim of this study is to evaluate the association of 45 SNPs with BPD susceptibility in a Turkish premature infant cohort.
METHODS: Infants with gestational age <32 weeks were included. Patients were divided into BPD or no-BPD groups according to oxygen need at 28 days of life, and stratified according to the severity of BPD. We genotyped 45 SNPs, previously identified as BPD risk factors, in 192 infants.
RESULTS: A total of eight SNPs were associated with BPD risk at allele level, two of which (rs4883955 on *KLF12* and rs9953270 on *CHST9*) were also associated at the genotype level. Functional relationship maps suggested an interaction between five of these genes, converging on *WNT5A*, a member of the WNT pathway known to be implicated in BPD pathogenesis. Dysfunctional *CHST9* and *KLF12* variants may contribute to BPD pathogenesis through an interaction with WNT5A.
CONCLUSIONS: We suggest investigating the role of SNPs on different genes which are in relation with the Wnt pathway in BPD pathogenesis. We identified eight SNPs as risk factors for BPD in this study. In-silico functional maps show an interaction of the genes harboring these SNPs with the WNT pathway, supporting its role in BPD pathogenesis.
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IMPACT:

- It is known that genetic factors may contribute to the development of BPD in preterm infants. Further studies are required to identify specific genes that play a role in the BPD pathway to evaluate them as a target for therapeutic interventions.
- Our study shows an association of BPD predisposition with certain polymorphisms on *MBL2*, *NFKBIA*, *CEP170*, *MAGI2,* and *VEGFA* genes at allele level and polymorphisms on *CHST9* and *KLF12* genes at both allele and genotype level.
- In-silico functional mapping shows a functional relationship of these five genes with *WNT5A*, suggesting that Wnt pathway disruption may play a role in BPD pathogenesis.

INTRODUCTION

Despite recent advances in neonatal intensive care, bronchopulmonary dysplasia (BPD) remains one of the most common cause of neonatal morbidity and mortality, due to the increasing survival of extremely low birth weight (ELBW) infants.[1,2] Birth weight and gestational age are the most important risk factors.[3,4] In addition, several maternal, perinatal, and postnatal factors including race, sex, infections, altered microbiome, mechanical ventilation, and oxygen injury all contribute to the complex pathogenesis of BPD.[2,5]

Although approximately 40% of ELBW infants develop BPD, the reported incidence varies widely between centers, not only as a consequence of definition criteria and clinical care practices, but also possibly reflecting innate differences in the populations studied.[3,4] In recent years, genetic susceptibility was increasingly recognized as a contributing factor for BPD development in premature infants. In a premature twin cohort study, genetic factors were estimated to account for 53% variance in BPD.[6] BPD development and severity are closely associated with both common and rare variants involving a vast array of genes, and outcomes are influenced by complex genetic and environmental interactions.[2,7] Rare variants are often unique, thus offering little diagnostic value, but inform on significant genes and pathways. In recent years, genome-wide association studies (GWAS) attempting to determine BPD candidate genes through the identification of common and rare deleterious variants in several preterm infant

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Table 1. rs numbers, chromosome locations, gene names, and allele information of investigated SNPs.

SNP ID	Chromosome location	Gene symbol	Alleles	Refs.	SNP ID	Chromosome location	Gene symbol	Alleles	Refs.
rs1001179	11p13	*CAT*	C/T	(20)	rs4883955	13q22.1	*KLF12*	G/T	(15)
rs10162694	15p7	*ZNF770*	A/C	(15)	rs55716084	1q43	*CEP170*	A/G	(15)
rs1049269	10q22.1	*SPOCK2*	A/G	(8)	rs5746136	6q25.3	*SOD2*	C/T	(20)
rs1049982	11p13	*CAT*	T/C	(20)	rs62468577	7q21.11	*MAGI2*	T/C	(15)
rs11003125	10q21.1	*MBL2*	C/G	(10)	rs650950	11p13	*PAMR1*	C/T	(15)
rs11226613	11q22.3	*CARD17*	A/T	(15)	rs6988306	8q22.3	*GRHL2*	C/T	(15)
rs1126214	18q12.1	*DSC3*	C/T	(15)	rs699947	6p21.1	*VEGFA*	A/C	(13)
rs1245560	10q22.1	*SPOCK2*	C/A	(8)	rs78975256	5q23.1	*PRR16*	C/T	(15)
rs12701220	7p22.3	*CYP2W1*	C/T	(15)	rs8192287	4p15.2	*SOD3*	G/T	(20)
rs1440306	4q26	*NDST4*	G/T	(15)	rs833061	6p21.1	*VEGFA*	C/T	(13)
rs1474256	15q25.1	*RASGRF1*	C/T	(15)	rs9877396	3p14.2	*FHIT*	C/T	(15)
rs1541364	5q31.2	*SPOCK1*	A/G	(15)	rs9953270	18q11.2	*CHST9*	C/T	(15)
rs17551536	2p24.3	*MIR3681*	A/G	(15)	rs2278034	3q29	*TNK2*	C/T	(15)
rs17880135	21q22.11	*SOD1*	G/T	(20)	rs769217	11p13	*CAT*	C/T	(20)
rs1912816	8q23.3	*CSMD3*	G/A	(15)	rs7096206	10q21.1	*MBL2*	C/G	(9)
rs2536512	4p15.2	*SOD3*	A/G	(20)	rs71627250	5q12.3	*HTR1A*	C/T	(15)
rs2664349	8q21.3	*MMP16*	A/G	(22)	rs72791417	10q21.3	*CTNNA3*	A/T	(15)
rs2664352	8q21.3	*MMP16*	C/T	(22)	rs755622	22q11.23	*MIF*	C/G	(14)
rs3138053	14q13.2	*NFKBIA*	C/T	(12)	rs1966265	5q35.2	*FGFR4*	G/A	(21)
rs349423	1p34.2	*HIVEP3*	C/T	(15)	rs204732	21q22.11	*SOD1*	G/A	(20)
rs3771150	2q12.1	*IL-18RAP*	A/G	(23)	rs2233406	14q13.2	*NFKBIA*	A/G	(12)
rs3771171	2q12.1	*IL-18R1*	C/T	(23)	rs2233409	14q13.2	*NFKBIA*	A/G	(12)
rs4634985	10p12.31	*PLXDC2*	T/C	(15)					

cohorts showed associations of certain single nucleotide polymorphisms (SNP) on genes such as *ACE*, *MBL2*, *NFKBIA*, *TNF*, *VEGFA*, *SPOCK-2*, and *TLR10* with BPD development,[8–15] but yielded inconsistent results, with some studies reporting no significant association with any SNP.[15,16] The majority of these variants were related to lung development, drug metabolism, and immune response.[15] A recent whole-exome sequencing (WES) study in a cohort of 85 infants with extreme severe respiratory phenotypes[17] revealed both rare and common variants of 292 genes, 19 of which replicated the findings of a previous WES study,[18] and the relation between BPD and various key signaling pathways like gonadotropin and corticotropin-releasing hormones (lung maturation), PKA-cAMP (respiratory epithelial cell differentiation), cardiovascular hypertrophy (cardiac or pulmonary vascular dysfunction) and EGFR/Neuregulin (surfactant, BPD phenotype) were emphasized.

In this study, we sought to validate the correlation between previously identified common allelic variants (SNPs) in 45 selected sites associated with BPD in a Turkish cohort of very premature infants. We then performed bioinformatics pathway analysis in order to explore the potential relationships and pathways involved between the affected genes.

MATERIAL AND METHODS

Study design

We designed a prospective cohort study comparing the prevalence of selected SNPs between subjects with and without BPD. The study population consisted of 192 infants hospitalized in Kanuni Sultan Suleyman Training and Research Hospital, Neonatal Intensive Care Unit between December, 2016 and March, 2018. Infants born under 32 weeks of gestational age were eligible, excluding those with major congenital anomalies and/or whose parents declined to participating were excluded. Survivors were evaluated for BPD at 28 days of age based on oxygen requirement >21% and divided into two groups (BPD, no-BPD) on a 1-to-1 ratio after obtaining parental consent. The study was planned based on an estimated sample size of 194 subjects (assuming a 20% difference between groups, alpha 0.05, beta 0.2). BPD patients were subsequently stratified into three groups of severity (mild, moderate, severe) based on supplemental oxygen levels and need for mechanical ventilatory support at 36-weeks postmenstrual age according to the 2001 National Institute of Child Health and Human Development (NICHD) consensus statement.[19] During the study period, standardized protocols based on both European[20] and Turkish[21] guidelines were followed for antenatal, delivery room, and NICU care by the same team for all study population. Peripheral blood samples were collected by one investigator after enrollment. The study received the approval of the Ethics Committee of Kanuni Sultan Suleyman Training and Research Hospital (KAEK/2016.12.31).

Selection of relevant SNPs

We performed an extended review of the literature through PubMed and found a number of reported associations with surfactant proteins, lung and vascular development genes, adhesion molecules, antioxidant enzymes, inflammation-related genes, and matrix modeling proteins in different populations. We selected 45 SNPs based on a systematic review of adequately powered genomic studies reporting significant associations with BPD.[8–16,22–26] These SNPs are located on genes related to oxidases, destruction of free superoxide radicals, zinc-finger proteins, calcium and mannose-binding proteins, certain growth factors, interleukins, inflammation response, and embryonic development. Physical positions, chromosomal locations, gene and allele information are provided in Table 1.

Experimental methods

All methods used in this study were carried out according to iPLEX® Gold Application Guide[27] and MassARRAY® Assay Design 3.1 Software Guide[28] (Sequenom Inc., San Diego, CA) unless otherwise specified. QIAamp DNA Mini Kit (Qiagen GmbH, Dusseldorf, Germany) was used to isolate leukocyte DNA; HotStarTaq plus DNA polymerase, PCR buffer, and $MgCl_2$

Table 2. A: An example of primer design for rs1049269 polymorphism on *SPOCK2* gene. B: List of all primers used in this study.

(A) DNA sequence of rs1049269 polymorphism [A/G]			**Length**
5′TCCACAG**GGAGCTGCTGTTTTGTCTAC**CTTTTGAGGGGACCAGTCTCAGT(A/G) GGGCGAAAGGCAGTGTGG*GGCTTCGGAAACAGGCAAGG*CTGGACCCCTG3′			50 bp [A/G] 50 bp
(B) SNP ID	**Forward primer sequence**	**Reverse primer sequence**	**Extended primer sequence**
rs833061	ACGTTGGATGAGTGAGGACGTGTGTGTCTG	ACGTTGGATGTTGGAATCCTGGAGTGACCC	TCTCCCCGCTCCAAC
rs12701220	ACGTTGGATGTGCAGCTTTAAGCCATCCGC	ACGTTGGATGCACCCCACCACCCCGAGAA	CCTGCAAACTGCTCG
rs2278034	ACGTTGGATGACCCCACTGTCCCCAAAAAC	ACGTTGGATGCCTGGTGTCCCTAACTCATC	ACAGAACAGCAGCCT
rs755622	ACGTTGGATGCGATTTCTAGCCGCCAAGTG	ACGTTGGATGTCCAGCAACCGCCGCTAAG	GCCAAGTGGAGAACAG
rs71627250	ACGTTGGATGCCTGTGTCCTAAGTTTTGAG	ACGTTGGATGGGAGATTGTTAAGTAGAACC	GACCCAGTTGCCAATTC
rs55716084	ACGTTGGATGACAGACTTGCTGCCTATATG	ACGTTGGATGGAGAACTTTCCATGTCAACC	AGCTGACCTTTAACTGA
rs1049269	ACGTTGGATGGGAGCTGCTGTTTTGTCTAC	ACGTTGGATGCCTTGCCTGTTTCCGAAGC	CCACACTGCCTTTCGCCC
rs6988306	ACGTTGGATGTCTGGTGCAGATGTTCCAAA	ACGTTGGATGGGAATCAGGCTCACTACATC	cccCAACCTGTACTGCCC
rs1966265	ACGTTGGATGGTCCCTGAGAGCTGTGAGAA	ACGTTGGATGAGAGGCCTCCAGGGACAAGA	agaGCACACTCAGCAGGA
rs1541364	ACGTTGGATGCTCACTTCCCTAGGGGTTAG	ACGTTGGATGGGGAAGGAACTGTGAACTTG	ttccGCCTGTCCAAGTTCC
rs3771150	ACGTTGGATGATGTCAACATGACCCTTAGC	ACGTTGGATGGCAAAGTGCAGGCTATTATC	cattACCCTTAGCCCCGGT
rs1126214	ACGTTGGATGCCTGTAAGCTCCCCAAATTC	ACGTTGGATGACATTGAGGGAAAAGTTCA	GTGAGTTTTTCTTGCTCTG
rs650950	ACGTTGGATGACATCCTGGATTCTGTTTAC	ACGTTGGATGGAACTGATGGTTCAGCTTGG	gaAGCTTGGCATTTCCCAAA
rs4883955	ACGTTGGATGTGCAGATGTCAGTTACCAAC	ACGTTGGATGTTTTCTAAAGGCTGACTTG	gaacGGCTGACTTGAGAACA
rs1245560	ACGTTGGATGTTTGCTTATGTTCTTGGGTG	ACGTTGGATGTTTGTGTGAGGCACCCTCG	GGGCCTCTGGGATGAAAAAG
rs8192287	ACGTTGGATGTACTTATGAGTGCGGCTAGT	ACGTTGGATGCCACCGTGAAGATCCAATGA	ccccCATTACTCGCCCAGTGA
rs72791417	ACGTTGGATGGTGTGTGGCATACACACAAT	ACGTTGGATGCTTCTCATTCTGCTTGCTTG	cccgCTGCTTGCTTGGCCTTT
rs7096206	ACGTTGGATGCTGCTGGAAGACTATAAAC	ACGTTGGATGCCTAAGCTAACAGGCATAAG	GGAAGACTATAAACATGCTTTC
rs2233406	ACGTTGGATGGCAAGGTGTTAATGTTTGTAG	ACGTTGGATGGTAGGTCAGATAGCATAAACG	ggatTGTGGATACCTTGCAATA
rs78975256	ACGTTGGATGTATAGCAGATGTAGCAGCAC	ACGTTGGATGACCATCTCTAAACAATACCC	AAACAATACCCTTTCTAAACTTC
rs699947	ACGTTGGATGTTCCCATTCTCAGTCCATGC	ACGTTGGATGAGTCAGTCTGATTATCCACC	ccggCTGATTATCCACCCAGATC
rs62468577	ACGTTGGATGTAAATGTGATAGGGATGTGC	ACGTTGGATGCTCAGACTCCAGAATAGATG	ggagCCAGAATAGATGGGACTAC
rs1912816	ACGTTGGATGCATCATACCCCATGTAAAAA	ACGTTGGATGGCCAAATGATTTTTCCTTT	TGATTTTTCCTTTAATTTGAAGAT
rs769217	ACGTTGGATGCATATACCTGTGAACTGTCC	ACGTTGGATGTTAGGCCTACCCTGATTGTC	aagcGTGGCCAACTACCAGCGTGA
rs11003125	ACGTTGGATGCTGGAGTTTGCTTCCCCTTG	ACGTTGGATGGTATTTAGCACTCTGCCAGG	tctcTTGCTTCCCCTTGGTGTTTTA
rs10162694	ACGTTGGATGGGTGCCCACAAGGATATTTG	ACGTTGGATGCAATTTACTCTTATCAGATGG	ATCAGATGGATAAAAATGAAAAATT
rs1001179	ACGTTGGATGCTGAAGGATGCTGATAACCG	ACGTTGGATGCAGCAATTGGAGAGCCTCG	cttAGCCCCGCCCTGGGTTCGGCTAT
rs11226613	ACGTTGGATGCTCATAGTGAAGAATTTCTC	ACGTTGGATGTGAAATGGGCCCAGGAGAG	agcAAAGTAACACAACCTTTAGAGAA
rs1440306	ACGTTGGATGCATAAGATGAAAATGTGCAG	ACGTTGGATGAAGCAAACGGAATTGCTGCC	ccctaCAATGCACTGCCTGCGGACAAC
rs1474256	ACGTTGGATGTGGGGAAAGATGCTCTGCAA	ACGTTGGATGAGGAAGCATTGCCTCCCAC	GGAACAGGGATGGGG
rs2233409	ACGTTGGATGCTGCACCCTGTAATCCTGTC	ACGTTGGATGCGACGACCCCAATTCAAATC	aTCCCTCTGCAAGTGA
rs3771171	ACGTTGGATGAGAGACAGGGTAGCAGATAG	ACGTTGGATGCTTTACTCTCATAGTGATCAG	TGTCCCAGAGTGGATA
rs9877396	ACGTTGGATGCCCAGAGAGATATTTGATCC	ACGTTGGATGCATTCTCATTGCTTGGGTGG	tGCTGGGAAGGATGTTT
rs2664352	ACGTTGGATGTAGCGAGGCTCATCAGGCA	ACGTTGGATGCCCAGATGTACATAGCTACC	ggCAAACTCCACTCACCT
rs5746136	ACGTTGGATGCAGAAGTATCACTGCAGAGG	ACGTTGGATGCACTTTCCCAAGGGAAACAC	TCACTGCAGAGGATTACT
rs204732	ACGTTGGATGTCTCCCCACATAAATGGTCC	ACGTTGGATGATCTGAGTCTAGTCCACAGC	CTGCTGTGAAGTTTTTGAG
rs1049982	ACGTTGGATGTGGAGACCCACGAGCCGAG	ACGTTGGATGAGTGCTGCATCTGGTCGCT	ggttTGCGGTTTGCTGTGC
rs2536512	ACGTTGGATGTGGCAGGAGGTCATGCAG	ACGTTGGATGACCCGGGGCTGCGCGGCGTC	gggaAGGCGGCGTGGAGCG
rs3138053	ACGTTGGATGGTGTTCATAAGTAGCTATTCG	ACGTTGGATGTCACGATCCTTTTTCTGCGG	TTTATGCTATCTGACCTACA
rs9953270	ACGTTGGATGGCCTGTTCAGTTCATTGTAT	ACGTTGGATGCTCAATACAAGTCTTTGCTG	GACTGCTTGGGTATCATCTTG
rs17551536	ACGTTGGATGGTCTTGAGAGCAAACGAGTC	ACGTTGGATGCATAGTGTGCAGCATATGG	ggatAAAGGAGTTGGCCTAAT
rs17880135	ACGTTGGATGGGAGAGGAAAAGCTAAATTGG	ACGTTGGATGGGTTGTTTTAACCACTTAC	tcTTTAACCACTTACTGTGAAC
rs4634985	ACGTTGGATGAAGTGCTGGGATTACAGTTG	ACGTTGGATGCTGAGGAAGGGATTCCTAAT	GTTGTTAAAAACCACACTGTTG
rs349423	ACGTTGGATGCAGCTTGGAATTGTTCTGTC	ACGTTGGATGAAATGCTCTGGGAAGCTCCG	GGAATTGTTCTGTCTATAAACTA
rs2664349	ACGTTGGATGTGTTGCAGTCTGCAGATAGG	ACGTTGGATGGACTATGCTTATCTCGAGAC	agggTCTCGAGACAGAACTTTTA
rs2786189	ACGTTGGATGGAGAAATAAGGAGGCTGTTG	ACGTTGGATGCTGTCAGCATCTCCTATTAC	tggGGCTGTTGAGGGTCT

Forward (bold), reverse (italic), and extension (underlined) primers are shown and the SNP site is indicated with brackets.

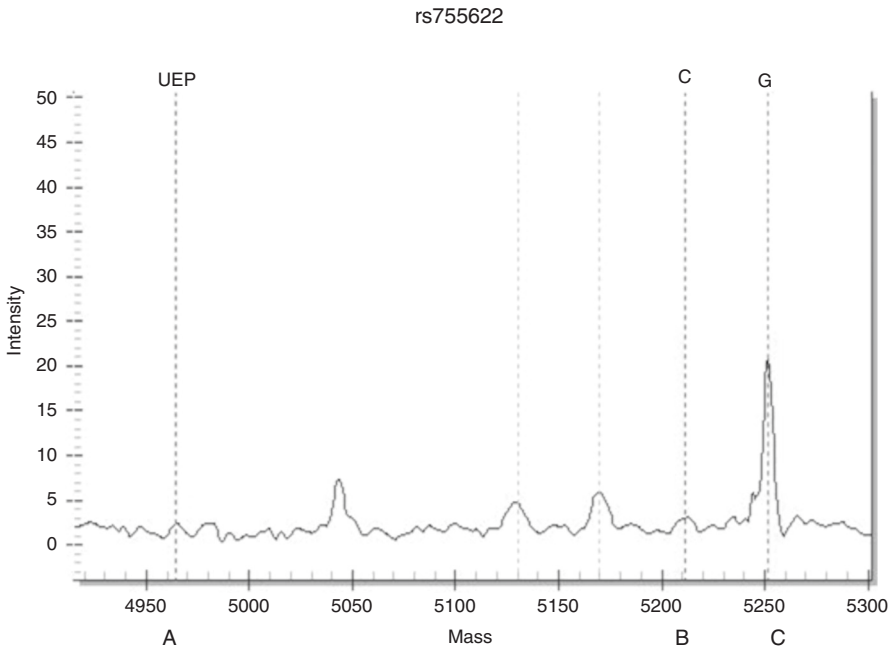

Fig. 1 Spectrum image of rs755622 polymorphism. a UEP, **b** Peak for C allele, **c** Peak for G allele.

solutions used in PCR were obtained from Qiagen, dNTP solution from Applied Biosystems, Inc (Foster City, CA). Shrimp alkaline phosphatase (SAP) enzyme and enzyme buffer solution, iPLEX enzyme, iPLEX buffer solution, and iPLEX Extension Mix used in single base extension (SBE) reactions, SpectroClean Resin solution, and primers for PCR were obtained from Agena Bioscience GmbH, Hamburg, Germany. DNA isolation was performed according to the manufacturer's instructions.[29] DNA concentrations were measured for each DNA sample by spectrophotometry. All DNA samples had an A_{260}/A_{280} ratio between 1.7 and 2.0 and concentrations higher than 10 ng/µl by 260/280 nm spectrophotometry,[30,31] hence were included in further analyses.

Primer and probe design

Positions of primers and extension probes on the genome sequence were determined by Agena Biosciences, Assay Suite bioinformatics software. Amplification primers were designed to include the polymorphic site as a multiplex PCR product and produce an 80–120 bp length amplicon. A label of 10 nucleotides (5′-ACGTTGGATG-3′) was added to the 5′ end of amplification primers. Extension probes were designed to have a different mass than amplification primers to avoid any confusion in the spectrum. Extension primers were designed to be 15–20 nucleotides long, with a T_m value of ≥60 °C, and able to anneal right next to the SNP site from its 3′ end (Table 2). All forward and reverse custom-designed primers and probes were manufactured by Agena GmbH.

Multiplex PCR

We used MassARRAY® Multiplex PCR (Agena GmbH) for the amplification of target SNP sites, and Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) fingerprinting method for genotyping SNPs, as accurate, rapid, and cost-effective for genotyping SNPs by multiplex assays in a single procedure. In brief, following the first PCR, SAP enzyme was used to neutralize dNTPs which were not bonded to the amplification products, by cutting the free phosphate group from dNTPs and turning them permanently into dNDPs as published. The PCR products were then hybridized with extension primers specifically designed for each SNP, and mass-modified SBE reaction was performed. SBE reaction products were then treated with resin to remove Na^+, K^+, and Mg^{2+} ions in order to reduce the background noise, and PCR products were transferred to 384-element SpectroCHIP® II by using a nanodispenser and was uploaded to the mass spectrometer (MassARRAY® Analyser 4, Agena GmbH) for analysis.[32] MassARRAY® Type 4.0 genotyping software was used to analyze data, obtain allele-specific peaks and spectrum images.[27]

Genotyping

The spectrum image of the rs755622 polymorphism is provided as an example in Fig. 1. The molecular weight (MW) of the extension primer designed for this polymorphism is 4964.3 Da. It is known that the C or G alleles added to this sequence with the iPLEX reaction increase the nucleotide MW to 5210.8 and 5250.5 Da, respectively. These three MW values are identified as A, B, and C peaks; the "A" peak corresponding to unexpanded primers (UEP) with a MW of 4964.3 Da, the "B" peak corresponding to the 5210.8 Da sequence of the C-extended allele, and the "C" peak corresponding to the 5250.5 Da sequence of the G-extended allele.

Statistical analysis

All statistical analyses were performed using SPSS software (Statistical Packages of Social Sciences, SPSS for Windows, Version 20.0, Chicago, IL). Results with OR > 1 and $p \le 0.05$ were considered statistically significant. Chi-squared tests, performed to compare observed vs. expected genotype frequencies for deviations from Hardy–Weinberg Equilibrium (HWE), showed that the control and BPD study groups were statistically in balance for 38 out of the 45 selected SNPs ($p > 0.05$), while deviating from HWE for seven (rs1049269, rs1966265, rs3771171, rs650950, rs72791417, rs769217, and rs8192287), which were excluded from subsequent analyses.

The major allele in the control group was referred to as the wild-type allele. Odds ratios (OR) of a minor (presumably pathogenic) vs. major alleles were calculated with a 95% confidence interval (CI). Associations between genotypes and BPD were determined in comparison with the prevalent genotype in the control group using both homozygote (e.g., AA vs. CC) and dominant (e.g., AA + AC vs. CC) comparison models. Sample size determination using the one-sided McNemar's test resulted in 192 individuals (96 in each arm) with 80% power and a 0.05 level of significance. Clinical data were reported by number and percentage of cases.

Functional relationship analysis

We generated functional relationship webs of the genes carrying the most significant BPD-associated SNPs at the allelic plus/minus genotypic levels in this cohort using an online bioinformatics tool computing various functional genomic datasets such as gene expression, cellular localization, and DNA/protein binding assays (PathwayNet, Troyanskaya Lab, Princeton University, Princeton, NJ).[33,34] The findings were validated using a tissue-specific functional genomic network tool (HumanBase, Flatiron Institute, Simons Foundation, New York, NY).[35]

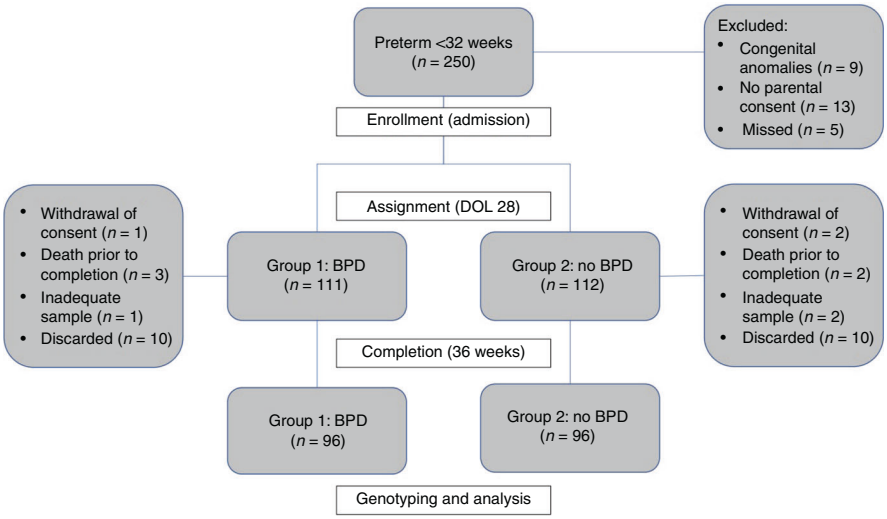

Fig. 2 Study flow chart. DOL day of life.

RESULTS

Demographic data

After initial exclusion, postnatal demise, consent withdrawal, and technical issues, a total of 192 infants constituted the study cohort. From these, 96 infants with a diagnosis of BPD were referred as the study group, while 96 infants without BPD referred to as the control group, as shown on the cohort flow chart (Fig. 2). In the study group, 44 (46%), 34 (35%), and 18 (19%) infants had mild, moderate, and severe BPD, respectively (Table 3). The study group had significantly lower birth weight and gestational age compared with the control group (both $p < 0.001$) (Table 3). Duration of invasive and non-invasive mechanical ventilation and oxygen exposure was significantly higher in infants with BPD compared with the control group. There were no significant differences in terms of sex, antenatal steroid status, breastmilk feeding, neonatal morbidities, or mortality ($p > 0.05$).

Association of BPD predisposition and SNPs at the allele level

Eight polymorphisms (rs11003125, rs2233406, rs3138053, rs55716084, rs62468577, rs833061, rs4883955, and rs9953270) harbored in seven genes (*VEGFA, CHST9, NFKBIA, CEP170, MAGI2, MBL2*, and *KLF12*) were associated with BPD risk, since the prevalence of the risk alleles in these SNPs was statistically higher among the BPD subjects than among the no-BPD group (Table 4A).

Association of BPD predisposition and SNPs at the genotype level

Two out of 38 polymorphisms resulted associated with BPD at the genotype level. The TT genotype of *rs4883955* and the CC genotype of *rs9953270* polymorphisms were significantly enriched in the BPD compared to the control group [OR = 5.48 (1.48–20.33), $p = 0.01$ and OR = 2.71 (1.02–2.58), $p = 0.04$ respectively] (Table 4B). Statistical results for polymorphisms that were not found to be in an association with BPD in this study are available in the Supplementary Information.

Functional relationship of genes associated with BPD

The functional relationship map performed for *VEGFA*, *CHST9*, *NFKBIA*, *MBL2*, and *KLF12* indicated direct functional interactions between four out of five of these genes (except *CHST9*) with weak to moderate confidence. In addition, the map revealed a novel functional convergence of these five genes on another gene, WNT5A, with moderate to strong confidence. In particular, CHST9 and KLF12, the two genes associated with BPD at both allele and genotype level, were directly and independently related with WNT5A with confidence indices of 0.34 (*WNT5A-CHST9)* and 0.6 (*WNT5A-KLF12)*. The map also suggested a functional connection of four of the five BPD-associated genes within another gene, PHLDA1, although with a weaker confidence index (Fig. 3a). Tissue-specific pathway analysis focusing on the 3 genes (*WNT5A, KLF12*, and *CHST9*) in lung (Fig. 3b) and fetal tissue (Fig. 3c) confirmed the findings for the *WNT5A-KLF12* (evidence 0.13 for lung and 0.06 for fetal tissue) and *WNT5A-CHST9* interactions (0.06 for lung and 0.07 for fetal tissue). Due to analyzing limitation of 5 genes in PathwayNet tool, CEP170 and MAGI2 genes were excluded from the relationship web shown in Fig. 3a, as PathwayNet and HumanBase bioinformatics tools do not show any association of these genes with WNT5A.

DISCUSSION

BPD remains the most frequent complication of prematurity, resulting from the complex interactions of antenatal and postnatal environmental exposures with an innate background of the developing lung. BPD risk and long-term outcomes are determined by the sequential influence of maternal and fetal conditions, acute injury and repair, and healing and remodeling processes, at each step of which individual gene variation plays a key modulating role.[1,36] Major risk factors for BPD include prematurity and intrauterine growth restriction. In fact, if 80% of infants born at 22–24 weeks of gestation will develop BPD, only 20% of infants born at 28 weeks will do so.[1] In concordance with the literature, infants who developed BPD in our study had significantly lower gestational age and birth weight. Hyperoxia and mechanical ventilation are also recognized as other important risk factors for BPD.[37] Indeed, duration of invasive or non-invasive mechanical ventilation and total oxygen exposure were significantly higher in infants with BPD in our study. Several postnatal comorbidities such as ventilator-induced lung injury, infection/sepsis, patent ductus arteriosus, and nutritional deficiencies may also influence the onset and severity of BPD. In order to minimize the impact of these variables, standard delivery room and NICU management policies have been developed for BPD prevention and treatment.[38,39] Herein, in order to mitigate the confounding effect of these risk factors and limit treatment-related disease heterogeneity, strictly standardized care protocols were implemented for life support and comorbidities management in this cohort.

Table 3. Comparison of the case and control groups for demographic features and neonatal morbidities.

	BPD (+) ($n = 96$)				BPD (−) ($n = 96$)				
	Mean ± SD/*n*%			Median	Mean ± SD/*n*%			Median	*p*
Sex									
Female		50	52.10%			51	53.10%		0.83
Male		46	47.90%			45	46.90%		0.86
Birth weight (g)	1041.4	±	262.7	1080	1358.9	±	353.9	1430	**<0.001**
Gestational age (weeks)	28.6	±	2.4	29	29.9	±	2.2	30	**<0.001**
Chorioamnionitis		4	4.17%		3		3.13%		0.70
Antenatal steroid		76	79.20%		75		78.10%		0.89
RDS		96	100%		90		93.80%		0.19
Duration of invasive MV (days)		2.72 ± 2.77			0.78	±	1.76		**<0.001**
Duration of NIV (days)		23.78 ± 12.5			9.65	±	8.34		**<0.001**
Total 02 (days)		67.84 ± 38.50			18.73	±	8.72		**<0.001**
Mostly BM (>80%)		80	83.3%		82		85.4%		0.12
NEC		15	15.60%		12		12.50%		0.23
hsPDA		38	39.58%		34		35.42%		0.55
Sepsis		14	14.60%		17		17.70%		0.56
ROP		30	31.30%		26		27.10%		0.53
PHT		18	18.75%		11		11.46%		0.16
IVH		9	9.40%		8		8.30%		0.80
IUGR		5	5.21%		4		4.17%		0.73
Mortality		9	9.40%		4		4.20%		0.15
Mild/moderate/severe BPD	44 (45.8%)	34 (35.4%)	18 (18.8%)		-		-	-	

Mann–Whitney *U* test/t-test/Chi-square test.
SD standard deviation, *BPD* bronchopulmonary dysplasia, *RDS* respiratory distress syndrome, *BM* breastmilk, *NIV* non-invasive ventilation, *MV* mechanical ventilation, *hsPDA* hemodynamically significant patent ductus arteriosus, *PHT* pulmonary hypertension, *IUGR* intrauterine growth restriction, *NEC* necrotizing enterocolitis, *IVH* intraventricular hemorrhage, *ROP* retinopathy of prematurity.
Bold values indicate statistical significance $p < 0.05$.

Table 4. A: Association of SNPs and BPD at allele level. B: Association of selected SNP's and BPD at the genotype level.

(A) SNP				Frequency of prevalent allele		Frequency of minor allele		OR and *P*-values of minor allele according to the prevalent allele	
SNP ID	**Gene**	**1. allele**	**2. allele**	**BPD**	**Control**	**BPD**	**Control**	**OR (95% CI)**	***P*-value***
rs11003125	*MBL2*	G	C	0.559	0.626	0.440	0.374	1.318 (1.10–1.57)	0.0023
rs2233406	*NFKBIA*	G	A	0.659	0.715	0.341	0.285	1.298 (1.07–1.57)	0.006
rs3138053	*NFKBIA*	T	C	0.647	0.703	0.353	0.297	1.295 (1.07–1.65)	0.006
rs4883955	***KLF12***	**G**	**T**	**0.649**	**0.741**	**0.351**	**0.259**	**1.547 (1.27–1.87)**	**<0.0001**
rs55716084	*CEP170*	G	A	0.973	0.995	0.027	0.005	4.945 (3.73–6.72)	<0.0001
rs62468577	*MAGI2*	C	T	0.798	0.868	0.202	0.132	1.675 (1.32–2.13)	<0.0001
rs833061	*VEGFA*	C	T	0.484	0.607	0.516	0.393	1.648 (1.38–1.97)	<0.0001
rs9953270	***CHST9***	**T**	**C**	**0.566**	**0.656**	**0.434**	**0.344**	**1.462 (1.22–1.75)**	**<0.0001**

(B) SNP	Gene	Sample size	Genotype	BPD	Control	OR (%95 CI)	*P*-value*
rs4883955	*KLF12*	BPD	GG	41 (0.471)	45 (0.517)	Reference	
		87	GT	31 (0.356)	39 (0.448)	0.872 (0.46–1.64)	0.67
		Control	TT	15 (0.172)	3 (0.034)	5.487 (1.48–20.33)	**0.01**
		87	GT + TT	46 (0.528)	42 (0.482)	1.202 (0.66–2.17)	0.54
rs9953270	*CHST9*	BPD	TT	29 (0.318)	37 (0.397)	Reference	
		91	CT	45 (0.494)	48 (0.516)	1.196 (0.63–2.25)	0.57
		Control	CC	17 (0.186)	8 (0.086)	2.711 (1.02–7.15)	**0.04**
		93	CT + CC	62 (0.681)	56 (0.602)	1.412 (0.77–2.58)	0.26

*$P \leq 0.05$

Bold values indicate statistical significance $p < 0.05$.

In A, all SNPs that are found to be associated with BPD at allele level are shown. The rows marked with bold indicate that these two SNPs are found to be associated with BPD at both allele and genotype level, as it can be also seen in B.

BPD is also, at least in part, a heritable condition. Twin studies suggested the possible role of genetic contribution on the development of BPD. Although genetic variation has been extensively studied in BPD, most studies have failed to identify rare variants consistently present in affected infants, and the strongest and most frequent associations have been found with common variants (SNPs). Several studies revealed functional mechanisms by which SNPs, located both in protein-coding and non-coding genes, affect gene transcription (mRNAs), regulation (miRNAs), splicing, stability, and post-transcriptional processes, thus contributing to the pathophysiology of various multifactorial diseases and allowing the identification of candidate genes playing a role in organ development function and homeostasis.[40,41] In particular, SNPs studies in the BPD population have highlighted the importance of genes involving lung development, fibrosis, maturation, oxidative stress, inflammation, angiogenesis, or tissue injury and repair.[42] In general, most of these studies focused on relatively few genes or pathways and were based on limited samples and populations (one or few centers). Most studies focusing on rare variants failed to identify a single -or a small cluster of- candidate genes associated with BPD.[18] One of the first GWAS studies on BPD allowed to identify one critical gene (*SPOCK2*) and novel pathways including adenosine deaminase and targets of miR-219,[43] but was not consistently reproduced in subsequent studies, making a call for more clinical and translational studies aimed at elucidating the role of genetics in BPD development. In another pilot study, five rare and novel candidate genes (*MMP1*, *TLR1*, *NOS2*, *CRP*, and *LBP*) were identified in severe BPD infants by next-generation sequencing.[44] Subsequently, several other candidate rare variants were identified in two large WES studies (including *FBN1, DAG1, MRPL16, POLR3A, PPP1R3A, NCAM1, SLC38A2, GABRA2, SOX6*).[17,18] However, connecting and integrating these data from various sources and identifying central pathophysiological pathways remains a major challenge. Our study highlights the association of variations in five genes with BPD susceptibility, in particular *CHST9* and *KLF12*, and suggests a functional interaction of these genes with the Wnt pathway.

CHST9 gene encodes a protein that belongs to the sulfotransferase 2 family, which is located on the Golgi membrane, catalyzing the transfer of sulfate to position 4 of non-reducing *N*-acetylgalactosamine (GalNAc) residues in N-glycans and O-glycans.[45,46] These sulfate groups on carbohydrates play essential roles in cellular interactions, signal transmission, and embryonic development as they can augment glycoprotein, glycolipids, and proteoglycan functions. In human, *CHST9* is associated with acute myelogenous leukemia, schizophrenia, breast, and gastric cancer.[47–49] It is highly expressed in the trachea and fetal lung,[48,49] but no evidence of any relation between *CHST9* and BPD or other lung diseases has been reported to our best knowledge.

Krüppel-like factors (KLF) belong to a family of zinc-finger transcription factors playing key roles in cellular development, metabolism, differentiation, and activation.[50] Currently a total of 18 mammalian KLFs were reported to be expressed in various tissues and during periods of development.[50] KLF12 induces the programmed cell death process (anoikis) by supporting the G1/S transition in the cell cycle. *KLF12* silencing in mouse models leads to increased tumoral lung cell survival.[51] Another KLF family member, KLF5, modulates genes regulating surfactant lipid and protein homeostasis, vasculogenesis (including *VEGFa),* and smooth muscle cell differentiation, and plays an important role in perinatal lung morphogenesis and function.[52]

The WNT gene family consists of structurally-related genes that encode intercellular signaling proteins. Canonical WNT/β-catenin signaling is essential for lung development in utero. *WNT5A*$^{-/-}$ mice die immediately after birth out of hypoxic respiratory failure, due to defective alveolar-vascular coupling and interstitium thickening.[53] Fetal WNT5A inhibition is associated with abnormal branching of distal airways together with defects in capillary and

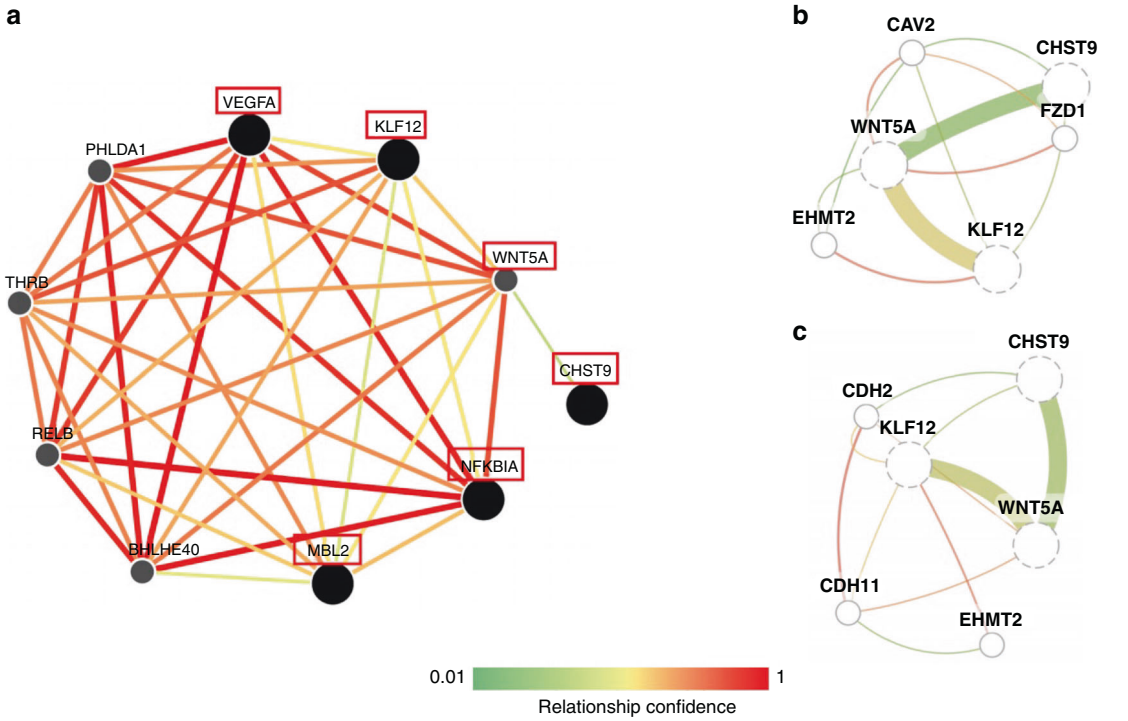

Fig. 3 Functional relationship web maps of BPD-associated genes identified in the cohort. a Functional relationships of KLF12, CHST9, VEGFA, MBL2, and NFKBIA human genes (pathwayNet, http://pathwaynet.princeton.edu). **b**, **c** Tissue-specific relationships of KLF12 and CHST9 with WNT5A in lung (**b**) and fetal tissue (**c**) (HumanBase, https://hb.flatironinstitute.org).

distal airspace formations.[54,55] *WNT5A* overexpression in distal lung epithelium also leads to epithelial branching defects and dilated distal airways,[56] suggesting a complex balance in WNT ligands and receptors time and age-related expression patterns, and tightly regulated signal dosages leading to specific sequential effects in lung development, maturation, and regeneration.[57] Intra-amniotic lipopolysaccharide exposure, an established BPD model, decreases canonical WNT signaling in the developing lung of preterm lambs, differently modulated by corticosteroids according to the timing of treatment.[58] During alveologenesis, WNT signaling activation leads to Type 2 alveolar epithelial cell (AEC) proliferation whereas WNT inhibition promotes epithelial maturation and transdifferentiation of type 2 to type 1 AEC.[59] *WNT5A* regulates branching morphogenesis during the pseudoglandular stage and promotes and AEC differentiation during lung maturation from the onset of the saccular stage.[60] Increased mesenchymal WNT5A during saccular-stage hyperoxia injury leads to alveolarization impairment and septal thickening similar to a BPD phenotype.[61] In postnatal life, WNT/β-catenin signaling plays a critical role in lung injury repair processes,[62] as observed in chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and asthma.[54,57,63,64] Postnatal WNT5A inactivation in a conditional loss-of-function mouse model results in alveologenesis interruption resembling BPD, and WNT5A expression is decreased in human BPD lung samples.[65] In summary, multiple evidences concur to demonstrate the central role of the WNT pathway in airway development, function, and disease.

Recently WNT5A emerged as a key mediator of pulmonary endothelial-pericyte interaction. WNT5A loss leads to pulmonary arterial hypertension by reducing the viability of newly formed vessels in animal and human.[66] A dysregulation of WNT signaling system exists in BPD-associated-pulmonary hypertension.[67] This finding may also be important as 40% of preterm infants suffering from severe BPD also suffer from BPD-associated pulmonary hypertension, with a significant contribution to morbidity and mortality.[67] KLF12 and WNT5A collaborate for the regulation of metabolic processes of nitrogen compounds.[33,34] Nitric oxide (NO) plays important regulatory roles in pulmonary vascular endothelium and airway epithelial functions during development and postnatally.[68] Data from our group and others' show that quantitative expression and distribution pattern of NO synthase isoforms are altered in BPD.[68–70] Thus, we can speculate that KLF12-related disruption of the NO pathway is a potential mechanism contributing to BPD pathogenesis,[68] but functional studies of KLF12 disruption fall beyond the scope of this project. To our knowledge, there are not any published data supporting the hypothesis of a KLF12 dysregulation in BPD-associated pulmonary vascular disease.

PHLDA1 encodes an evolutionarily conserved proline-histidine-rich nuclear protein with epithelial cell tumor-suppressing properties, highly expressed in the lung.[71] Although transcriptomics studies have shown a mirror alteration of PHLDA1 and WNT5A in smokers and COPD patients, suggesting a functional interaction between the two genes in lung disease, no data are available in BPD to date,[72] hence we opted not to focus on this gene.

In summary, the role of the WNT signaling pathway is increasingly recognized in the pathogenesis of BPD,[73] and our data suggest a possible role of CHST9 and KLF12 interaction with WNT5A in this process.

Our study presents some important limitations. Several disease severity indices appear higher in our cohort than in other published studies. As a single-center cohort study, our findings may be influenced by genetic assets of the Turkish population, antenatal and perinatal management of the fetus, and pregnancy (our center is a safety-net public hospital caring for underserved population), and center-specific obstetrics and neonatology practices. Thus, more clinical studies are needed to validate our findings in different populations and environments. With our approach of testing a set of SNPs identified in other cohorts, this study may fail to identify new variants specific to our cohort. Thus, an unbiased genomic approach such as next-generation sequencing might have yielded different or broader findings. Such approach was beyond the scope of the current project but could be pursued as a next step in a broader cohort. In addition, bioinformatic genome-scale functional maps of human issues, by integrating data sets covering several thousands of experiments, may yield proofs of concept, but they should be complemented with molecular experiments in order to demonstrate these specific functional relationships, understand these complex pathway interactions in the precise context of disease, and identify molecular targets for future interventions.

In conclusion, this study conducted in a single center with standardized, evidence-based clinical practices further supports the contribution of common genetic variants in BPD susceptibility, in addition to the well-established roles of environmental factors and comorbidities.

Functional in-silico mapping of these BPD-associated genes informs on specific molecular pathways potentially relevant for pathogenesis. We highlight the functional interdependence of these genes with *WNT5A*, a key player in lung morphogenesis, homeostasis, and injury repair both in utero and postnatally, suggesting the implication of its pathway in the pathogenesis of BPD. This study illustrates the potential of functional relationship analysis in SNP studies in order to identify novel therapeutic targets for the prevention and cure of BPD.

A deeper analysis of Wnt pathway genetic variation in BPD should include a broader list of genes in larger cohorts from different ethnic group. Functional in-vitro or in-vivo studies may be necessary in order to validate the effect of these polymorphisms on *WNT5A* expression and how it affects the nitric oxide pathway in BPD patients. Moreover, as the effects of common genetic variation on outcome in various conditions affecting the neonate are increasingly emerging, genome-scale polymorphism studies are warranted in order to explore the combined effects of multiple SNPs on BPD pathogenesis.

DATA AVAILABILITY

All authors accept that all data support their published claims and comply with field standards.

MATERIAL AVAILABILITY

All authors accept that all materials support their published claims and comply with field standards.

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AUTHOR CONTRIBUTIONS
A.A.; conceptualization, methodology, acquisition of data, formal analysis and investigation, resources, and writing—original draft preparation, S.Y.S; conceptualization, methodology, acquisition of data, and writing—original draft preparation, O.M.U.; formal analysis and investigation, and resources, A.E.; formal analysis and investigation, and resources. M.C.; conceptualization, methodology, acquisition of data, and writing—review and editing. O.D.; writing—review, editing, and providing the final version of the manuscript. D.T.-B.; conceptualization, methodology, acquisition of data, formal analysis and investigation, resources, and writing—original draft preparation, funding, and supervision.

ETHICS APPROVAL
The study was approved by the local Ethics Committee of Kanuni Sultan Suleyman Training and Research Hospital (KAEK/2016.12.31).

COMPETING INTERESTS
The authors declare no competing interests.

CONSENT TO PARTICIPATE
Written and signed informed consent was taken from all parents of all included infants.

CONSENT FOR PUBLICATION
All authors approve and give consent for this version to be published.

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