

# NKX2-5 molecular screening and assessment of variant rate and risk factors of secundum atrial septal defect in a Moroccan population

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## ABSTRACT

**Objective:** Secundum atrial septal defect (ASDII) has multifactorial etiology that is combination of environmental (e.g., mother's exposure to toxicity, ethnicity) and genetic causes. Aim of the present study was to screen a Moroccan population with ASDII for NKX2-5 variants and to assess risk factors that may contribute to emergence of the disorder.

**Methods:** Thirty-two non-syndromic ASDII patients were screened for NKX2-5 variants using direct sequencing of polymerase chain reaction-amplified coding regions. Risk factor rates were compared to general population and assessed using Fisher's exact and chi-square tests. In this retrospective study, criteria of exclusion were suggestive or confirmed syndrome association.

**Results:** Three heterozygous variants were detected in 4 patients. NKX2-5 variant rate in present cohort is estimated to be about 9.4%. Two prominent risk factors in the Moroccan population were highlighted: consanguinity, rate of which was significantly high at 30.8%, and previous maternal miscarriage or sibling sudden death, observed in 34.6% of cohort.

**Conclusion:** Impact of identified variants was discussed and possible disease-predisposing effect is suggested. Findings indicate that ASD may be favored by consanguineous marriage and that NKX2-5 variant rate in ASD patients may be affected by ethnicity. High level of maternal miscarriage and sibling sudden death suggests potential non-sporadic nature as result of putative genetic defect. (*Anatol J Cardiol* 2017; 17: 217-23)

**Keywords:** atrial septal defect, genetic screening, Moroccan population, NKX2-5, risk factors, variant rate

## Introduction

Congenital heart defect (CHD) is considered the most common congenital malformation, affecting 1% of live births and 10% of involuntarily aborted fetuses (1, 2). Secundum atrial septal defect (ASDII) accounts for approximately 10% of CHDs and 85% of all ASDs (3, 4).

ASDII is characterized by communication between right and left atrial compartments caused by malformation of the septum primum, which leads to incomplete coverage of the ostium secundum (5).

This disorder is result of multifactorial etiology involving both genetic and environmental factors (e.g., mother's exposure to toxicity, ethnicity) (6). Several studies have suggested association between ASDII and deficit of transcription factors involved in cardiogenesis such as NK2 homeobox 5 (NKX2-5), GATA-binding protein 4 (GATA4) and myosin heavy chain 6 (MYH6).

Whereas syndromic ASDII has been reported to be related to chromosomal abnormalities or deficit in specific syndrome-causing genes such as T-box transcription factor (TBX5) deficit in Holt-Oram syndrome or Ellis-van Creveld gene (EVC) deficit in Ellis-van Creveld syndrome (7).

NKX2-5 is a highly evolutionarily conserved homeobox gene expressed in the developing heart and it plays a leading role in progenitor specification, chamber formation, and conduction system development, among other aspects (8–10).

Several pathogenic variants of NKX2-5 have been identified in a wide range of congenital heart diseases, including septation defects and tetralogy of Fallot (11, 12). However, ASD with or without atrioventricular block is still the most frequent type of CHD caused by NKX2-5 pathogenic variants. The frequency of NKX2-5 variants in sporadic cases of ASD was estimated to be between 1% and 4%, while in familial cases it was reported to be about 8% (11, 13–15).

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In this study, variant screening of NKX2-5 in 32 Moroccan patients with non-syndromic ASDII was conducted with the aim of assessing, for the first time, the spectrum of variants as well as risk factors for Moroccan population with ASDII. The second goal was to compare the prevalence of NKX2-5 variants in our cohort to previous studies carried out in different populations.

## Methods

### Study subjects

The 34 patients initially enrolled in this study were seen at the cardio-pediatrics department and confirmed to have ASDII, with defect diameter most often greater than 5 mm. Clinical cardio-pediatric diagnosis comprises physical examination, electrocardiography, and color Doppler echocardiography. All patients were interviewed in order to assess disease history and obtain pedigree. Two patients with suggestive syndromic ASDII were excluded. This retrospective study was approved by the Institution Ethics Committee.

### Molecular analysis

After obtaining informed, written consent, blood samples were obtained from 32 non-syndromic ASDII patients. Genomic deoxyribonucleic acid (DNA) was extracted from blood leukocytes using optimized salting-out method (16). The 2 coding exons of NKX2-5 were amplified by polymerase chain reaction (PCR) using 3 pairs of primers derived from published data (17). As the second exon was long, it was divided onto 2 overlapping fragments, 2.1 and 2.2.

PCR was performed in a 25 mL mix volume containing 40 ng of genomic DNA, 1 U of Taq (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA), 20 pmol of each primer, 15–25 mM MgCl<sub>2</sub>, 10 mM dNTP, and 1X PCR buffer (Invitrogen) in Veriti 96-Well Thermal Cycler 9902 (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA). PCR cycling conditions were 94°C for 7 minutes followed by 40 cycles of 94°C for 40 seconds; 59°C, 62°C, and 64°C, respectively for exon 1, exon 2.1, and exon 2.2 for 30 seconds; 72°C for 40 seconds; and 72°C for 7 minutes.

Purified PCR products were sequenced using BigDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA) and run on Applied Biosystems 3500Dx Genetic Analyzer. Chromatogram was analyzed using Sequencing Analysis SeqA software version 5.4 (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA). Sequences thus obtained were analyzed with bioinformatics analysis tool of NCBI, the nucleotide BLAST alignment program (<http://blast.ncbi.nlm.nih.gov>).

### Statistical analysis

To compare different rates between study cohorts we used Fisher's exact test for reduced cohort sizes and chi-square tests for larger cohort sizes. Statistical computations were performed using the R software package (Free Software Foundation, Boston, MA, USA).

## Results

### Clinical investigation of cohort study

In this study, 32 unrelated patients with non-syndromic ASDII were enrolled for NKX2-5 variant screening. The cohort included 19 females and 13 males. Their ages range from 3 months to 21 years with a median of 4 years and an average of around 6 years. All patients were Moroccan with 2 major ethnic origins, Arabic (68.4%) and Amazigh (26.3%).

In this cohort, consanguinity rate was 30.8%, and averages of maternal and paternal age were respectively 27 and 36 years. Almost thirds of studied patients (34.6%) have at least 1 incident of early-infant sibling sudden death or previous maternal spontaneous abortion.

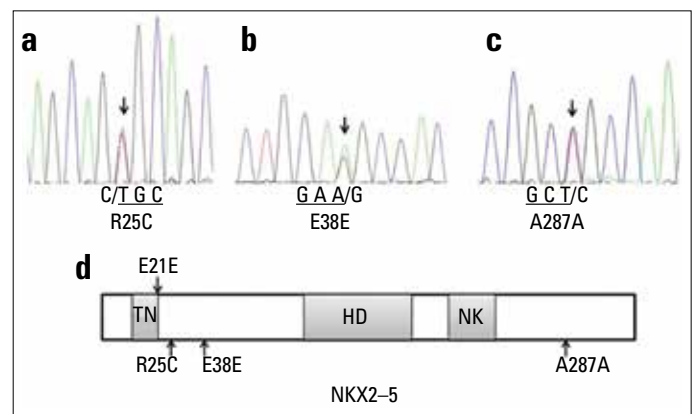
### NKX2-5 variants

In the present study, molecular screening of NKX2-5 coding exons in 32 patients with ASDII allowed us to identify 3 heterozygous variants in 4 patients (Table 1). Missense variant c.73C>T (rs28936670) was found in 3 participants, P1, P2, and P3. In the latter patient, a second variant, c.114G>A (rs151314714), was also observed, while the third variant, c.861C>T (rs77612903), was identified in patient P4. NKX2-5 variant rate in our cohort is estimated to be 9.4%, and when considering silent variants, percentage rises to 12.5%.

Moreover, molecular screening revealed 2 polymorphisms. The c.335-69T>C found in patient P1 is a novel polymorphism located in the non-coding region preceding the second exon. The c.63A>G (rs2277923) is a widespread variant, and was seen in 56.25% of our cohort. Figure 1 and Table 1 provide additional details about variant screening results.

### Comparison of variant rate

Statistical comparison of variant rate (9.4%) with previous similar studies carried out in different ethnic ASD populations revealed a significant difference with cases in Yunnan Chinese



**Figure 1.** Sequencing chromatogram of the detected NKX2-5 variants, (a) Variant c.73C>T identified in patients P1, P2 and P3, (b) Variant c.114G>A identified in P 3, (c) Variant c.861C>T identified in P4, (d) organisation of NKX2-5 domains with exonic variants sites  
HD - homeodomain; TN - tinman domain

population and study comprising Australian and American patients. On the other hand, no significant difference was noticed when comparing R25C variant rate of our study and Lebanese ASD population. When comparing c.63A>G (rs2277923) variant rate in previous studies, we found no apparent significant difference. These findings are illustrated in Tables 2 and 3.

## Discussion

In this study we report for the first time NKX2-5 variants found in a series of Moroccan patients with non-syndromic ASD. Association of deficiency in this highly conserved homeobox factor

with non-syndromic ASD was first proven by Schott et al. (18), and several studies have confirmed this fact through identification of more NKX2-5 variants in several ASD populations (Table 2). Thus, in the second part of this work, we compared our variant rates to these different populations. In order to have a full view of ASD etiology, we also studied also some risk factors that may contribute to occurrence of CHD.

### Assessment of risk factors

Sex ratio in present cohort is 0.68, reflecting a relatively high incidence of ASDII among females. This ratio is very close to those previously reported, notably by Cascos et al. (19) and

**Table 1. NKX2-5 variants identified among 32 patients with secundum atrial septal defect**

Variation ID	Chromosome localization	Nucleotide substitution	Amino acid substitution	Exon/ Intron	Variant type	Patients	Frequency (%)
rs2277923	5:172662024	c.63A>G	E21=	Exon 1	SNP	18 patients	56.25
rs28936670	5:172662014	c.73C>T	R25C	Exon 1	Missense	P1, P2, P3	9.4
rs151314714	5:172661973	c.114G>A	E38=	Exon 1	Synonymous	P3	3.13
Novel variation	5:172660281	c.335-69T>C	–	Intron 1	SNP	P1	3.13
rs77612903	5:172659686	c.861C>T	A287=	Exon 2	Synonymous	P4	3.13

SNP - single-nucleotide polymorphism

**Table 2. Comparison of NKX2-5 variant rate across diverse populations with non-syndromic secundum atrial septal defect**

Region	Population reference	Study reference	NKX2-5 variant frequency in ASD patients (%)	P*
North Africa and Middle East	Lebanese	Abou Hassan et al. 2015 (45)	6/25 (24)	0.16
	Egyptian	Hussein et al. 2009 (46)	2/8 (25)	0.25
Europe	German	Stallmeyer et al. 2010 (12)	2/17 (11.7)	1
	Italian	Sarkozy et al. 2005 (47)	3/29 (10.3)	1
America and Australia	American	McElhinney et al. 2003 (15)	3/71 (4.2)	0.37
	Australian/American	Elliott et al. 2003 (14)	1/102 (0.9)	0.04
Asia	Chinese (Yunnan)	Yu Cao et al. 2016 (48)	0/105 (0)	0.01
	Chinese (Han)	Liu et al. 2011 (49)	3/58 (5.17)	0.66
	Japanese	Hirayama-Yamada et al. 2005 (50)	3/16 (18.7)	0.38
Morocco	Moroccan	Present study	3/32 (9.4)	–

\*Compared to present study rate using Fisher's exact test

**Table 3. Comparison of c.63A>G (rs2277923) genotype among secundum atrial septal defect populations**

Study reference	Population reference	[A] (A/A) frequency	[G] (G/G or A/G) frequency	P*	A/G** frequency	G/G** frequency (%)	P*
Yu Cao et al., 2016 (48)	Chinese	34/105 (32.4%)	71/105 (67.6%)	0.23	43/71 (60.6%)	28/71 (39.4)	0.17
Posch et al., 2008 (52)	German	69/170 (40.6%)	101/170 (59.4%)	0.74	82/101 (81.2%)	19/101 (18.8)	0.73
Present study	Moroccan	14/32 (43.75%)	18/32 (56.25%)	–	14/18 (77.8%)	4/18 (22.2)	–

\*Compared to present study rate using Fisher's exact test for smaller cohorts and chi-square test for large cohorts. \*\*This column and the adjacent right column indicate respectively the number of heterozygous and homozygous cases among the ASD population carrying the c.63A>G genotype. The percentages show, respectively, the frequency of the carriers of the homozygous and the heterozygous c.63A>G. these columns respectively: Frequency of heterozygous c.63A>G ; Frequency of homozygous c.63A>G

Throne et al. (20): 0.64 (p=0.88) and 0.5 (p=0.5), respectively. Rate of consanguineous marriage is relatively high, reflecting socio-cultural specificities characterizing Moroccan life and community relations. Presently, prevalence of consanguinity in the general population is reported to be about 15.25% (21). Compared to high consanguinity rate observed in ASDII cohort, which reaches 30.8%, difference was statistically significant (p=0.04). This finding allowed us to conclude that ASD seems to be favored in consanguineous marriages. This conclusion confirms previous studies carried out in Lebanese (22, 23), Saudi (24), and Indian (25) populations regarding ASD and CHD more broadly, and also supports hypothesis suggesting involvement of autosomal recessive genetic factors.

Averages of observed maternal and paternal age in our cohort were 27 and 36, respectively. Parental age is said to most likely have no effect on ASD. In fact, Su et al. (26) have thoroughly assessed effect of paternal age on CHD as well as different subtypes, including ASD, through studying data of about 1,89 Million Danish patients classified in different age categories (<20, 20–24, 25–29, 30–34...), but other than patent ductus arteriosus, they did not find any particular association.

Interestingly, we noticed high incidence of sibling death among proband families. Approximately thirds of studied patients (34.6%) have history of at least 1 incident of either previous maternal spontaneous abortion or early-infant sudden death among siblings. This reveals very high rate of under-5 morality among CHD patients compared to that reported by Moroccan ministry of health in the general population, which is limited to 30.5% (2011) (27). This finding suggests also that those patients may be non-sporadic cases, having putative inherited genetic defect that was responsible for siblings' sudden death. We could not verify this suggestion, however, as parents' DNA samples were not available.

On the other hand, this high lethality rate among siblings may be closely related to higher rate of consanguinity among the population, reflecting dependent factors. To confirm this hypothesis, we compared 2 groups within our cohort: those with positive consanguinity and positive sibling death and group with positive consanguinity and negative sibling death; however, no significant difference was found (p=1).

Due to limited size of present groups, this result may not be

precise. Authors recommend a more thorough study of larger cohort to properly assess link between consanguinity and sibling death history in ASD and CHD cohorts.

### NKX2-5 variants

Molecular screening of NKX2-5 coding exons revealed 3 heterozygous variants in 4 patients among the 32 patients with ASDII enrolled in this study. Missense variant c.73C>T (rs28936670) was detected in patients P1, P2, and P3. As previously reported, this substitution leads to R25C amino acid change, which is considered the most prevalent NKX2-5 variant in CHD (9, 28, 29). Codon R25 lying close to tinman domain (TN) is a conserved residue in several species, notably mammals (Fig. 2). Abnormal substitution of arginine with cysteine at this position alters the charge of this residue (basic to neutral). Akçaboy et al. (29) drew attention to the fact that although there is substitution of Q and E residue in NKX2-5 homologues of other organisms, these amino acids are thought to conserve the positive charge quality of this part of the NKX2-5 protein (Fig. 2).

Kasahara et al. (30) demonstrated that R25C NKX2-5 mutant exhibits impairment of ability to form dimers on dimeric DNA-binding site. Dentice et al. (31) proved significant functional defect of R25C NKX2-5 mutant through functional studies that revealed reduced transactivation and significantly impaired activity on target-gene promoters with a dominant-negative action on the wildtype NKX2-5 action. Neither study detected impairment in NKX2-5 DNA-binding as monomer.

NKX2-5 plays a crucial role in cardiac tissue at several stages of embryonic heart development, in which it is widely expressed. This transcription factor is known to be involved in cardiogenesis process regulation. It interacts with GATA4 and serum response factors among others in order to moderate expression of downstream target genes (32, 33). Previous studies reported that lack of NKX2-5 gene in murine embryos led to death caused by defects in developing heart tube (33, 34). Therefore, variants involved in impairment of NKX2-5 function are considered to be responsible for several forms of CHD, including ASD (18).

Although its pathogenicity has been proven in independent studies, effect of R25C is still the subject of debate. In some studies, R25C was also observed in control cohort and normal rela-

H. sapiens	1	--MFPSALTPTPFSVKDIILNLEQQQ <b>R</b> SLAAAGEL SARLEATLAP--SSCM	47	<a href="#">NP_004378.1</a>
P. troglodytes	1	--MFPSALTPTPFSVKDIILNLEQQQ <b>R</b> SLAAAGEL SARLEATLAP--SSCM	47	<a href="#">XP_518104.2</a>
M. mulatta	1	--MFPSALTPTPFSVKDIILNLEQQQ <b>R</b> SLAAAGEL SARLEATLAP--SSCM	47	<a href="#">XP_001096796.1</a>
C. lupus	1	--MFPSALTPTPFSVKDIILNLEQQQ <b>R</b> SL--AAGEL SARLEATLAP--ASCM	46	<a href="#">NP_001010959.1</a>
B. taurus	1	--MFPSALTPTPFSVKDIILNLEQQQ <b>R</b> SL--AAGEL SARLEATLAP--ASCM	46	<a href="#">NP_001039908.1</a>
M. musculus	1	--MFPSALTPTPFSVKDIILNLEQQQ <b>R</b> SL--ASGDL SARLEATLAP--ASCM	46	<a href="#">NP_032726.1</a>
R. norvegicus	1	--MFPSAL <b>T</b> HTPFSVKDIILNLEQQQ <b>R</b> SL--AAGDL SARLEATLAP--ASCM	46	<a href="#">NP_446103.1</a>
G. gallus	1	--MFPSP-VTTTPFSVKDIILNLEQQQ <b>Q</b> GGIAPMELSS-----PSCM	38	<a href="#">NP_990495.1</a>
D. rerio	1	MAMFSSQ-MTSTPFSVRDIILNLEQN <b>Q</b> EDM-VSLDMSQRLDSALIPTSSCM	48	<a href="#">NP_571496.1</a>
X. tropicalis	1	--MFASP-VTSTPFSVKDIILNLE-Q <b>H</b> QSGLSMPMDITSRLN-----SSCM	41	<a href="#">NP_001116891.1</a>

**Figure 2.** Multiple alignment of NKX2-5 protein sequences across species showing the R25 residue conservation among mammals

tives (35, 36), while in others it was segregated with disease and not detected in control cohort (9, 15, 28). Goldmuntz et al. (28) suggested that R25C is subject to ethnic variability, and Costa et al. (37) considered it a disease-modifying polymorphism. Akçaboy et al. (29) regarded it as a mutation with quite low penetration since only 25% of variant carriers had pathogenic cardiac phenotype, and Dentice et al. (31) considered this substitution a disease-predisposing variant (31).

These data were analyzed in light of recent guidelines for interpretation of sequence variants (38), and we concluded that as there is both benign and pathogenic evidence, R25C variant has what the guidelines define as uncertain significance.

In present study, 2 variants previously reported, c.114G>A (rs151314714) and c.861C>T (rs77612903), observed in patients P3 and P4, respectively, were identified. These substitutions are synonymous variants that conserve initial residues, corresponding respectively to E38 and A287. Nevertheless, they could have an earlier impact on alteration of splicing machinery (39, 40). We checked this hypothesis via Human Splicing Finder (HSF 3.0; <http://www.umd.be/HSF3/HSF.html>) (41) and found that these 2 silent variants constitute potential cause of splicing function impairment since they alter exonic splicing sites, which results in skipping the involved exon. On the other hand, Chevance et al. (42) proved in an interesting study that synonymous variants could be pathogenic, in the sense that they could affect translation speed, and thus disturb (i.e., reduce) expression rate. These findings suggest a possible pathogenic effect of the detected silent variants. Due to absence of functional studies as well as lack of data about silent variant frequency in healthy cohorts, it was difficult to categorize these variants according to the guidelines for interpretation (38).

Furthermore, we detected c.63A>G (rs2277923) variant, which was seen in 18 of 32 (56.25 %) patients. This silent variant (E21=) is located in the tinman domain and is considered a polymorphism since it is widespread among patients as well as control subjects (43, 44). Recent guidelines for sequence variants interpretation (38) consider such variant benign. In the present cohort, GG genotype was less frequent than AG genotype, 22.2% vs. 78.8%. It is worth mentioning that c.63A>C change leads to creation of Bpml restriction site (9). On the other hand, we found, interestingly, that this silent variant has potential impact on alteration of splicing function through both creation of exonic silencer splicing site and alteration of exonic enhancer splicing site. This data needs more investigation to be elucidated.

In addition to these exonic variants, we identified a novel variant in non-coding region, c.335-69T>C. We confirmed via Human Splicing Finder (41) that this variant does not disturb NKX2-5 splicing function, as it is not located within a potential consensus sequence involving splice sites.

It is worth noting that the absence of homozygous NKX2.5 is due mainly to the crucial role that this transcription factor plays during cardiogenesis. Indeed, loss of NKX2-5 gene in mice leads to embryonic lethality (34), which means that pathogenic homo-

zygous variant may lead to lethality in human embryos. This data may also explain significantly higher level of previous maternal spontaneous abortion history in present study.

### Comparison of variants rate

NKX2-5 variant rate among Moroccan ASD patients is about 9.4%. In the second part of this study, comparison of this rate with that of previous studies carried out in North African and Arab populations and with different populations from all over the world (12, 14, 15, 45–50) was made. No significant difference between rate of this study and those of Arab (45), North African (46), European (12, 47), American (15), Han Chinese (49), and Japanese (50) populations was found (Table 2). The only significant differences were observed in Yunnan Chinese population (48) and a study with both Australian and American patients (14), respectively ( $p=0.01$ ) and ( $p=0.04$ ). Based on these findings, authors suggest that NKX2-5 variant rate in ASD patients could be affected by ethnicity. These findings partly confirm remarks of Ping et al., who emphasized rarity of NKX2-5 variants in Chinese population with CHD (51).

We also compared R25C variant rate with previous studies and found, interestingly, that this variant is uncommon in ASD patients. Indeed, among all studies reported in Table 2, only Abou Hassan et al. (45) identified R25C in Lebanese ASD patients with a very close rate (8%) ( $p=1$ ). This variant was rather more common in tetralogy of Fallot patients (data not shown).

Finally, we compared c.63A>G (rs2277923) genotype with previous studies, and did not find significant difference between Moroccan, Chinese (48), and German (52) genotypes (Table 3).

### Study limitations

Given the absence of parents' DNA samples, we could neither determine whether the studied cases are sporadic or familial, nor identify segregation mode of detected variants. It would be pertinent to screen a larger cohort in order to obtain an authentic assessment of risk factors and variant rate in Moroccan population.

### Conclusion

To conclude, this study allowed us to emphasize, for the first time, genetic etiology in Moroccan ASD patients through NKX2-5 variant screening, and to assess risk factors that may enhance occurrence of this type of CHD in the population. We identified 3 heterozygous variants in 4 patients. We proved involvement of consanguinity in increasing ASD risk, and suggest possible effect of ethnicity factor in NKX2-5 variant rate. Finally, it would be interesting to screen, in larger cohort, other important transcription factors and genes that have been proven to play major role in heart development process such as GATA4. Such studies would provide a full view of genetic etiology of ASD in Moroccan population.

**Conflict of interest:** None declared.

**Peer-review:** Externally peer-reviewed.

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