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Prevalence/Incidence of Hereditary and Acquired Thrombophilia Markers among Egyptian Females with Recurrent Pregnancy Loss or IVF Failure

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Abstract

Background: The largest percentage of failed *in vitro* fertilization (IVF) cycles, are due to lack of implantation. As hereditary and acquired thrombophilia can cause in placentation failure, it may have a role in recurrent IVF failure.

Objective: Aim of this case-control study was to determine the most prevalent types of hereditary and acquired thrombophilia in women with recurrent IVF failures.

Materials and Methods: Case group comprised 2466 women, with a history of recurrent IVF failure. Control group was comprised of 531 men (Most prevalent DVT gender presented in our Clinics) suffering from Active DVT (To correlate the impact those factors IVF failure with an active thrombotic effect among the control group). All participants were assessed for the presence of inherited thrombophilias including: Factor V Leiden, methyl tetrahydrofolate reductase (MTHFR) mutation, prothrombin mutation, plasminogen activator inhibitor-1 (PAI-1) mutation, Factor XIII, ACE, HPA1, and APO E and the homocystein level, protein S and C deficiency, antithrombin III (AT-III) deficiency, lupus anti-coagulant, NK markers and anticardiolipin. Positive results are compared as regard most prevalent combinations.

Results: Having at least one thrombophilia known as a risk factor for recurrent IVF failure (95% CI=1.74-5.70, OR=3.15, p=0.00). Mutation of factor V Leiden (95% CI=1.26-10.27, OR=3.06, P=0.01) and homozygote form of MTHFR mutation (95% CI=1.55-97.86, OR=12.33, p=0.05) were also risk factors for recurrent IVF failure. However, we could not find significant difference in other inherited thrombophilia's.

Conclusion: Inherited thrombophilia is as prevalent in women with recurrent IVF failure compared men with multiple DVT. Homozygous APO E and ACE as well as heterozygous ACE, MTHFR A and PAI contribute as a major factor in patients with recurrent failure.

Keywords: Embryo implantation; Thrombophilia; Chromosomal abnormalities; Homozygous factors

Introduction

Despite the multiple embryo transfer in the majority of infertility centers, only one third of all *in vitro* fertilization (IVF) cycles reach clinically achieved pregnancy and the majority of them still fail [1]. The largest percentage of failed IVF cycles, are due to lack of implantation. In some patients, implantation failure occurs repeatedly [2]. Recurrent implantation failure (RIF) derives from the practice of IVF. The ability to identify implantation failure after the transfer of embryos raised the possibility that there exists a pathophysiological state leading to repeated implantation failure. RIF can be defined as the repeated lack of implantation after the transfer of embryos. The 2005 ESHRE PGD Consortium [3] defines recurrent implantation loss as ">3 embryo transfers with high quality embryos or the transfer of ≥ 10 embryos in multiple transfers; exact numbers to be determined by each center."

Embryo quality and endometrial receptivity are two significant factors that believed to be the key points in implantation. The possible causes of repeated embryo implantation failure have been widely investigated. The most identified causes are decreased endometrial receptivity, embryonic defects and factors with combined effects [4,5].

Established risk factors for recurrent IVF include anatomical, hormonal, immunological and chromosomal abnormalities in addition to inherited and acquired thrombophilia. In the majority of recurrent IVF failure cases, the cause remains unknown (idiopathic). Recently, the role of hereditary thrombophilia in recurrent IVF failure is implicated. The association between thrombophilia and recurrent pregnancy loss or poor pregnancy outcome is well known. It may act by impairing the initial vascularization process occurring at implantation, which is necessary for a successful pregnancy [6-9]. However, there are limited data on the association between thrombophilia, hereditary or acquired, with IVF failure [7,8]. Present study was conducted to determine the most prevalent thrombophilia factors to 2466 of female patients presenting to NSA lab during the period of 2007 to 2014.

Materials and Methods

After obtaining written consent from all participants, this prospective study was conducted between 2007 and 2014 in NSA laboratories, Egypt. Case group was comprised 2466 infertile women, aged 20-40 years old, with a history of recurrent IVF failure. Recurrent IVF failure was defined as at least three consecutive failed IVF cycles. Failed IVF cycle was defined as failure to achieve clinical pregnancy in a cycle in which at least three good quality embryos (grade I or II) were transferred. Indications for IVF were male factor, ovulatory factor, tubal factor and unexplained infertility.

Control group was comprised of Control group was comprised of 531 men aged between 20-70 years old suffering from Active DVT , presented to our clinics during the same period. This group is chosen as a guide to the trend of positive thromboembolic diseases in Egypt.

All participants were screened for antiphospholipid antibodies EUROIMMUN ELISAs for the determination of autoantibodies against phospholipids (Cat # EA 1621-9601) Protein C and S, and ATIII ELISA (R&D Systems Catalog Number DSPC10) This assay employs the quantitative sandwich enzyme immunoassay technique, Lupus anticoagulant Chromogenic Lupus Anticoagulant sensitive APTT reagent. Cephalin reagent with particulate activator (silica), freeze-dried. By Stago (Cat # 00599)

Inherited thrombophilia's including: factor V Leiden, methylene tetrahydrofolate reductase (*MTHFR) C677T* mutation, prothrombin *20210A* mutation, and plasminogen activator inhibitor-1 (PAI-1) mutation, Factor XIII, ACE, HPA1, and APO E. tests were performed using PCR method CVD strip assay ViennaLab CVD StripAssays® based on reverse-hybridization of biotinylated PCR using probes for variants and controls in a parallel array of allele-specific oligonucleotides followed by immobilized oligos on a test strip and results by enzymatic color reaction. Flowcytometry coulter Beckman elite is for CD16/56 PE/FITC monoclonal antibodies.

Statistical analysis

Numerical variables were reported as mean ± SD. We used independent sample t-test and Chi-square test to compare quantitative and qualitative variables, respectively. To evaluate whether inherited thrombophilia's could predict the recurrent IVF failure, we used logistic regression analysis. Univariable analysis was performed, in which odds ratio (ORS) and 95% confidence intervals (95% CIs) were calculated. P-value ≤ 0.05 was considered to be statistically significant. All analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 14.0, SPSS Inc, Chicago, Illinois, USA).

Results

This study was conducted to test the frequency of the associated causes of thrombophilia. The study included testing 2997 patients starting from 2007 till the end of 2014. Amongst the 2997 patients, 2,466 (82.3%) were women and 531 (17.7%) were men and served as a control for this study. The results acquired by the extraction of genomic DNA for the associated genes, CVD strip assay was then employed to confirm the presence of the disease the results were divided to positive, negative, and carrier. All percentages were accounted for and analyzed *via* excel with counts on the number of each category. Percentages of the frequency of inherited thrombophilia were subsequently derived; to compare the occurrence of inherited *vs.* acquired thrombophilia effectively. Flow-cytometric analysis for CD56+ and CD16+ was also carried out due to their correlation with inherited thrombophilia to further verify the results. The results below demonstrate generated graphs for all estimated frequencies, for the 2997 patients with tests that were aforementioned.

Prevalence of inherited thrombophilia among female patients

The graph below represents the percentage of Eleven-thrombophilia gene mutations associated with RPL in females. The thrombophilia markers included factor *V G1691A* (Factor V Leiden), factor II prothrombin *G20210A*, factor XIII V34L, b-fibrinogen, plasminogen activator inhibitor-1 (PAI-1), *GPIIIa L33P* (HPA-1a/b L33P), methylenetetrahydrofolate reductase *C677T* (*MTHFR C677T), MTHFR A1298C,* ACE I/D, Apo *B R3500Q*, and Apo E (E2,E3,E4).

Figure 1 demonstrated results that were predicted, as normal frequencies in female subjects within the population. The factors Prothrombin and APoB showed the highest negative percentages of approximately 95% or higher. Factors ApoE, and ACE demonstrated the highest prevalence in diseased individuals with percentages between 50-60%. Female carriers showed highest prevalence in *MTHFR A*, ACE, PAI with ranges of 45%-70%.

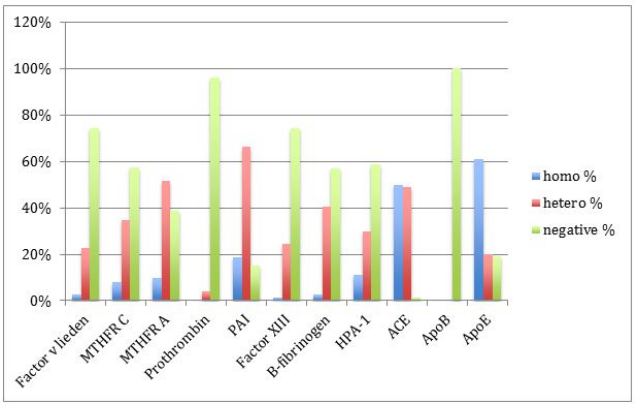

Figure 1: The genotype distribution of Eleven-thrombophilic gene polymorphisms in females.

Prevalence of inherited thrombophilia among male patients

The graph below represents the percentage of Eleven-thrombophilic gene mutation associated with RPL in males. The thrombophilic markers are factor *V G1691A* (Factor V Leiden), factor II prothrombin *G20210A*, factor *XIII V34L,* b-fibrinogen, plasminogen activator inhibitor-1 (PAI-1), *GPIIIa L33P* (HPA-1a/b L33P), methylenetetrahydrofolate reductase *C677T* (*MTHFR C677T), MTHFR A1298C*, ACE I/D, Apo B *R3500Q*, and Apo E (E2,E3,E4).

Which showed that Prothrombin and Apo B showed the highest negative percentages of approximately 95% or higher. Factors ApoE, and ACE demonstrated the highest prevalence in diseased individuals with percentages between 50%-60%. Female carriers showed highest prevalence in *MTHFR A*, ACE, PAI with ranges of 45%-70%. Due to the predominant variables amongst homozygous and heterozygous frequencies, allocation of gender specific frequencies were also generated with the same results they are represented in Figures 2-5.

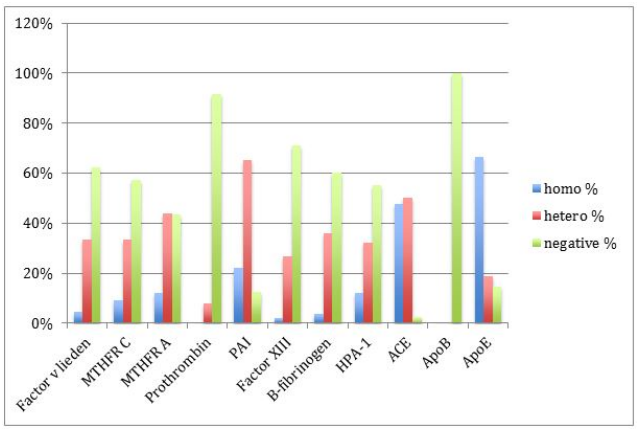

Figure 2: The genotype distribution of Eleven-thrombophilic gene polymorphisms in males.

Prevalence of Homozygous factors among both genders

The frequencies of homozygous mutations of the studied genes in the RPL and control males illustrated in Figure 5. The frequencies of homozygous mutations for factor *V G1691A,* MTHFR *C677T*, MTHFR *A1298C*, prothrombin *G20210A*, PAI-1, factor XIII *V34L*, b-fibrinogen, HPA, ACE I/D, Apo B , and Apo E were 2.89%, 8.12%, 9.89%, 0.09%, 18.66%, 1.31%, 2.61%, 11.29%, 49.72%, 0.00%, 61.01% respectively compared to control males which was 4.41%, 9.31%, 12.25%, 0.49%, 22.06%, 1.96%, 3.92%, 12.25%, 47.55% respectively.

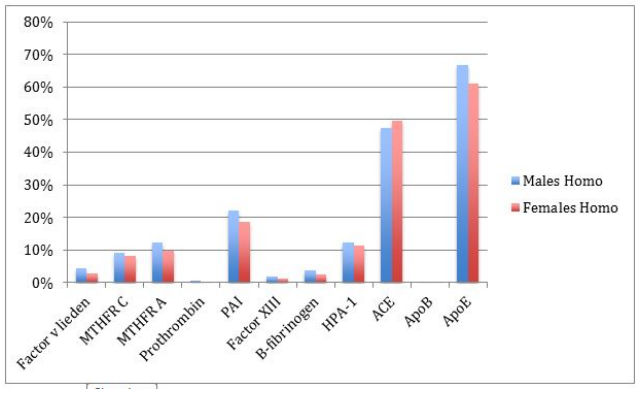

Figure 3: The frequencies of homozygous mutations in the RPL and control males for the studied genes (FV Leiden, *FV H1299R*, prothrombin *G20210A, FXIII V34L*, b-fibrinogen) 455G>A, PAI-1 4G/5G, *GPIIIa L33P*, *MTHFR C677T, MTHFR A1298C,* ACE I/D, Apo E).

The frequencies of homozygous mutations of the studied genes in the RPL and control males are presented in Figure 4. The frequencies of homozygous mutations for Apo E (Apo E4/Apo E3) were high in both RPL women compared with control males. The frequency of homozygous mutation for *MTHFR C677T* was 8.2 % in RPL women as it was nearly similar in control males 9.31%. As well as For MTHFR *A1298C*, plasminogen activator inhibitor-1 (PAI-1) the frequencies of homozygous mutations were similar in RPL women compared with the males as they were 9.89%, 18.66% compared to 12.25% and 22.06% respectively however. The frequencies of homozygous mutations for ACE I/D in RPL women and control males were quietly different as they were 18.66% compared to 47.55%.

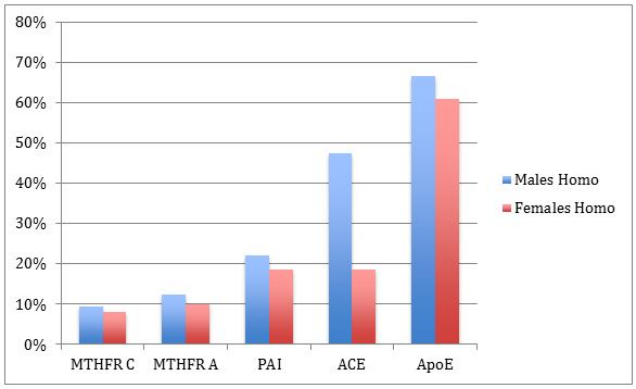

Figure 4: The frequencies of homozygous mutations in RPL women and control males which are affected with thrombophilia for Apo E (Apo E4/Apo E3), *MTHFR C677T, MTHFR A1298C*, PAI-1, ACE I/D.

The frequencies of heterozygous mutations of the studied genes in the RPL and control males illustrated in Figure 5 The frequencies of heterozygous mutations for factor *V G1691A, MTHFR C677T, MTHFR A1298C*, prothrombin *G20210A*, PAI-1, factor XIII V34L, b-fibrinogen, HPA, , ACE I/D, Apo B , and Apo E were 22.67%, 34.51%, 51.59%, 4.01%, 66.14%, 24.53%, 40.58%, 29.85%, 49.07%, 0.09% and 20.06% respectively compared to control males which were 33.33%, 33.33%, 44.12%, 7.88%, 65.20%, 26.96%, 35.78%, 32.35%, 50.00%, 0.00% and 18.63% respectively.

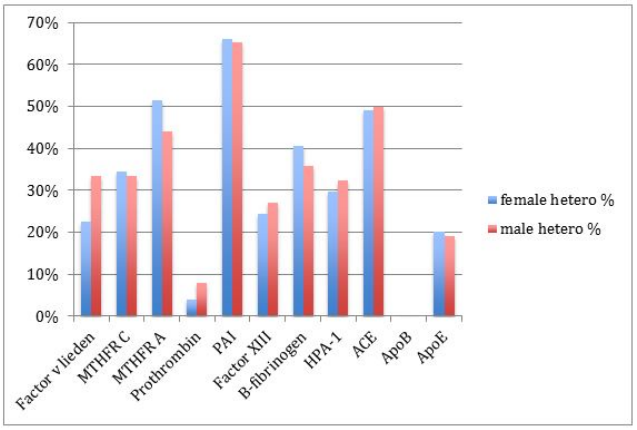

Figure 5: The frequencies of heterozygous mutations in the RPL and control males for the studied genes (FV Leiden, FV H1299R, prothrombin G20210A, FXIII V34L, b-fibrinogen) 455G>A, PAI-1 4G/5G, *GPIIIa L33P*, *MTHFR C677T, MTHFR A1298C,* ACE I/D, Apo E).

Prevalence of acquired thrombophilia among female patients

The above stated results were to highlight the prevalence of inherited thrombophilia, the second portion of this study focused on the assessment of acquired thrombophilia and its prevalence in the same number of patients Tables 1 and 2 displays results of ELISA on the anticoagulant proteins ACL IgG, ACL IGM, PtnC, PtnS, ATIII, LA and homocysteine, as well as the derived percentage of frequencies. Table 4 highlights males.

Tables 3 and 5 shows a summary of the results of flow-cytometery with the NK cell antigens CD56+ CD16+ which are known markers in females with inherited thrombophilia, and are known to cause recurrent implantation failures during attempted pregnancies.

	ACL IgG	ACL IGM	PtnC	PtnS	ATIII	LA	Homocysteine
positive %	1.31	3.18	0.47	0.19	1.21	2.05	0.75
negative %	98.69	96.82	99.53	99.81	98.79	97.95	99.25

Table 1: The percentages anticoagulants in females.

The percentages of ACL IgG, ACL IGM, PtnC, PtnS, ATIII, LA and homocysteine. Showing that the positive percentages of ACL IGM and LA showed the highest positive percentages of approximately 2% or higher.

	No. of +ve patients	Positive %	No. of –ve patients	Negative %
CD56+	20	0.93	2,446	0.07
CD16+	2	0.01	2,464	99.91

Table 2: The percentages of CD56+ CD16+ in females.

Table 2 shows a summary of the results of flow-cytometery with the NK cell antigens CD56+ CD16+ which are known markers in females with inherited thrombophilia, and are known to cause recurrent implantation failures during attempted pregnancies. The results showed that CD56+ CD16+ percentage in females was higher than that shown in the table below of males.

Prevalence of acquired thrombophilia among male patients

	ACL IgG	ACL IGM	PtnC	PtnS	ATIII	LA	Homocysteine
Positive %	1.47	0.98	1.47	0.98	0.98	1.47	6.86
Negative %	98.53	99.02	98.53	99.02	99.02	98.53	93.14

Table 3: The percentages of anticoagulants in males.

The results shown in Table 3 estimated the positive and negative percentages of the anticoagulants in males. Showing that the percentage of homocysteine was the highest compared to females and the LA, ACL IgG and PtnC was nearly the same between males and females.

	No. of +ve patients	Positive%	No. of –ve patients	Negative %
CD56+	0	0.00	531	100
CD16+	0	0.00	531	100

Table 4: The percentages of CD56+ CD16+ in males.

Table 4 shows a summary of the results of flow-cytometery with the NK cell antigens CD56+ CD16+ which are known markers in males with inherited thrombophilia, and are known to cause the risk of thrombosis. The results showed that CD56+ CD16+ negative percentages in males was higher than that shown in the Table below of females shown in Table 3. In A comparison between acquired factors, in women the ACL IgM was more prevalent in women than men and homocystien and Ptc are more prevalent in men.

Discussion

In vitro fertilization (IVF), which is mainly used for the treatment of infertility specifically in women over age 40, maybe offered in the following cases: Fallopian tube blockage or damage, Ovulation disorder, Premature ovarian failure, Endometriosis, Uterine fibroids, Previous tubal sterilization or removal, Impaired sperm production or function, Unexplained infertility, a genetic disorder. However there are some risks in IVF treatment including Multiple births, Premature

delivery and low birth weight, Ovarian hyper stimulation syndrome, Egg-retrieval procedure complications, Birth defects, Ovarian cancer, Stress, miscarriage.

The risk of miscarriage might increase with the increase of maternal age, the occurrence of this problem in 8% to 20% of the cases happen before 20 weeks of growth of the baby when in fact 80% of the occurrence happen in the first 12 weeks of pregnancy, Moreover RPL may be caused by thrombophilia in 40% to 50% of the cases.

Thrombophilia diagnosis is carried out through testing the Eleven-thrombophilia gene mutation associated with RPLAs as well as testing the anticoagulants. The thrombophilia markers are factor *V G1691A* (Factor V Leiden), factor II prothrombin *G20210A*, factor XIII *V34L*, b-fibrinogen, plasminogen activator inhibitor-1 (PAI-1), *GPIIIa L33P* (HPA-1a/b L33P), methylenetetrahydrofolate reductase *C677T* (*MTHFR C677T), MTHFR A1298C*, ACE I/D, Apo B *R3500Q*, and Apo E (E2,E3,E4).

In this study, the Eleven-thrombophilia gene mutations associated with RPL were investigated in the heterozygous cases and were compared to the control (males). The percentage of factor V G1691A, represented by (22.67%), was much lower than that of the control indicated by (33.33%), *MTHFR C* represented (34.51%) was slightly higher than the control (33.33%), *MTHFR A* was (51.59%) which is higher than the control (44.12%), prothrombin *G20210A* was 4.01% which is lower than the control (7.88%), PAI-1 was (66.14%) which is higher than the control (65.20%), factor XIII was (24.53%) which is lower than the control indicated by (26.96%), while b-fibrinogen was (40.58%) which was higher than the control (35.78%), HPA was (29.85%) which was lower than the control (32.35%), ACE was indicated by (49.07%) which is lower than the control (50.00%), Apo B was indicated by 0.00% which is similar to control (0.00%), Apo E represented by (20.06%) which was higher than the control (18.63%).

Upon comparing the detected homozygous cases with the control (males) with active DVT in order to be positive that those genetic and acquired factors are associated with an active thrombophilia effect, the percentage of factor V G1691A was represented as (2.89%) which was lower than that of the control, which was (4.41%). *MTHFR C* had an incidence of (8.12%) which is slightly lower than the control represented by (9.31%), *MTHFR A* was (9.89%) which is lower than the control (12.25%), meanwhile, prothrombin *G20210A* was 0.09% which is lower than the control (0.49%), PAI was (18.66%) which is lower than the control (22.06%), factor XIII (1.31%) was lower than the control indicated by (1.96%), b-fibrinogen was 2.61% which is lower than the control (3.92%), HPA was (11.29%) which is slightly lower than the control represented by (12.25%). ACE had an incidence of (49.72%) which is slightly higher than the control (47.55%). The incidence of Apo B was (0.00%) which was exactly the same as the control (0.00%), while Apo E was (61.01%) which is lower than the control (66.67%).

A study, which was performed at Department of Cellular and Molecular Biology, Biological Science Faculty, Azerbaijan Shahid Madani University, Tabriz, Iran, showed that the prevalence of heterozygous gene mutation of FV G1691A polymorphism in venous thromboembolic disease (DVT) patients was (8.89%) which is relatively lower than that reported in this study (22.67%), on the other there was no incidence for the homozygous gene mutation of the same gene (0.0%) while in this study was (2.89%), however it was also reported that there is a positive association between *FV G1691A* polymorphism with DVT, meaning that carriers of *FV G1691A* and prothrombin *G20210A* polymorphisms have an increased risk of deep venous thrombosis. Conversely, the frequency of prothrombin *G20210A* polymorphism of the heterozygous gene mutation was (16.66%) which is significantly high if compared to that reported by this study, while for the homozygous gene mutation; the percentage was (2.23%) which is higher than that reported in this study (4.01%). The prevalence of PAI heterozygous gene mutation was (30%) which is much lower than that reported in this study (66.14%). More over the homozygous gene mutation which incidence, indicated by (44.45%), is significantly high if compared to the percentage reported in this study (18.66%) [10].

A study was performed on the Combination of Thrombophilia Gene Polymorphisms as a cause of increased the risk of recurrent pregnancy loss giving the prevalence of the 4 thrombophilia polymorphisms in females stating the homozygous and heterozygous percentages of each gene giving that the homozygous percentage of the 4 genes was factor *V Leiden, PAI, MTHFR C* and *MTHFR A* which was represented by 1%, 9%, 15% and 4% respectively in comparison with this study which was 2.89, 18.66%, 8.12% and 9.89% the study results were extremely lower than this study in all genes except for the *MTHFR C* which was represented by 15% in the study and was higher than this study, moreover the heterozygous percentages for the 4 genes was 12% for factor V Leiden which was lower than this study and was represented by 22.67%, PAI was 9% and was extremely lower than stated in this study which was 66.14%, as well as *MTHFR A* was 27% and was also lower than stated in this study. Conversely, *MTHFR C* percentage was 42% which was higher than stated this study was 34.51% [11].

According to other studies that was performed in Department of Family Medicine, Cayiralan State Hospital, Yozgat, Turkey showed that The frequencies of homozygous mutations of b-fibrinogen, *HPA, PAI-1* and *MTHFR C677T* were 6.2%, 12.5%, 21.7% and 10.8% respectively which is higher than the reported in this study 2.61%, 11.29%, 18.66% and 8.12%. While, the frequency of prothrombin *G20210A* polymorphism of the heterozygous gene mutation in this study was 7% which was relatively higher than this study (4.01%), however the *FV Leiden* gene mutation was 19.1% which is lower than the reported in this study (22.67%). [12].

According to the tests performed in Egypt in Departments of Obstetrics and Gynecology Benha Faculty of Medicine, Benha showed that there are increases in the percentages of cases with heterozygous and homozygous mutations in *FV* gene (heterozygous 60% and homozygous 10%) was extremely higher than stated in this study which is represented by (heterozygous 22.67% and homozygous 2.89%), Moreover, the percentage of PTH gene mutation is also higher than this study which is heterozygous 35% and homozygous 30% compared to heterozygous 4.01% and homozygous 0.09% and *MTHFR C* gene was also higher in the heterozygous gene mutation which was represented by heterozygous 45% compared to 34.51% in this study and homozygous 25% compared to 8.12%. [13].

According to studies on Different Risks of Thrombosis in Four Coagulation Defects Associated With Inherited Thrombophilia: A Study of 150 Families done by Martinelli I. in 2015 stated that Deficiency of the naturally occurring anticoagulant proteins, such as antithrombin, protein C and protein S, and activated protein C resistance due to the factor V Leiden gene mutation is associated with inherited thrombophilia. 12% of the studied patients has antithrombin deficiency, 9 % of the studied patients has a deficiency in protein C and 6 % has deficiency in protein S. The risk of thrombosis induced by each

of these coagulation defects has been investigated by family studies, or case-control studies that showed an increased thrombotic risk in carriers of the defect compared with noncarriers. while according to this study it was stated that 0.47% of the patients had protein C deficiency, 0.19% had protein S deficiency and 1.21% of the patients had antithrombin deficiency which was relatively low compared to this study. [14].

A study which was performed by Department of Obstetrics and Gynecology, Vienna University Medical School on Genetic Polymorphisms Associated With Thrombophilia and Vascular Disease in Women With Unexplained Late Intrauterine Fetal Death stated that The deficiencies of natural anticoagulants are rare in the general population, as they stated that the positive percentage of the protein C deficiency, protein S deficiency and antithrombin are equal to 5% which was higher than was stated in this study which was 0.47% protein C deficiency in females, 0.19% protein S deficiency and a 1.21% antithrombin in females. [15].

As stated by the Department of Clinical Epidemiology University Hospital, Leiden, Netherlands on the Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study that The deficiencies of natural anticoagulants are rare in the general population and, combined, are found in less than 15% of all individuals and stated that the percentage of the deficiency of the anticoagulants among 800 females was Protein C deficiency was 5% which was exactly the same for protein S and antithrombin which in correlation with this study is much higher and was represented by 0.47% for protein C, 0.19% for protein S and 1.21% for antithrombin. [16].

Difference between the prevalence of genetic factors among different studies in different geographical arears might be multifactorial including hereditary, as well environmental including pollution stresses and sedentary *vs.* active lifestyle.

Conclusion

Inherited thrombophilia is as prevalent in women with recurrent IVF failure compared men with multiple DVT. Homozygous APO E and ACE as well as heterozygous ACE, *MTHFR A* and PAI contribute as a major factor in patients with recurrent failure.

Recommendation

A study comparing the outcome of those IVF trials in correlation with those results and the impact of anticoagulant uptake during the IVF process and throughout pregnancy.

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