ANTI-FORSSMAN PREVALENCE IN A SAMPLE OF THE PORTUGUESE POPULATION AND ITS CHARACTERIZATION

PREVALÊNCIA DE ANTI-FORSSMAN NUMA AMOSTRA DE POPULAÇÃO PORTUGUESA E A SUA CARACTERIZAÇÃO

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Conflitos de interesse
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Resumo

Introdução
Descoberto em 1911 por Frederick Forssman, a expressão do antigénio (Ag) Forssman (Fs) varia entre espécies, estando raramente presente nos eritrócitos humanos. Em 1987, três famílias inglesas sem qualquer relação entre si, foram identificadas com um fenótipo designado *Apae* que, mais tarde, foi classificado como o 31º grupo sanguíneo: FORS. Os anticorpos (Ac) anti-Fs têm ocorrência natural em humanos e podem ter implicações transfusionais e de transplante, uma vez que o Ag está presente na superfície de eritrócitos, fluídos corporais e órgãos.

Objetivos
O principal objetivo deste trabalho foi avaliar a prevalência de Ab anti-Fs e esclarecer o seu impacto na medicina transfusional, classificando o tipo de imunoglobulina (Ig) envolvida.

Materiais e Métodos
Neste estudo foi utilizada a técnica standard de tubo para avaliar a presença de Ac anti-Fs em amostras de plasma de uma população de dadores Portugueses e classificar as imunoglobulinas envolvidas. Foi utilizada, para a realização de todas as experiências, uma suspensão de eritrócitos de ovelha a 3-5% com expressão positiva para Ag Fs.

Resultados
De um total de 11877 amostras, 117 (0,99%) apresentaram reações fracas (entre 0 e 1 numa escala de 0 a 4) quando em contacto com o Ag Fs presente na suspensão de eritrócitos de ovelha. Essas amostras foram posteriormente estudadas quanto à presença da mutação no gene *GBGT1*, responsável pela expressão da enzima Fs sintetase. Das 192 amostras estudadas (50 de cada grupo sanguíneo, à exceção do AB – o mais raro), para classificar o Ac envolvido, 52% revelaram ser apenas IgM, sendo as demais, uma mistura entre IgG e IgM.

Conclusão
Na população estudada, poucas amostras (<1%) apresentaram reação negativa contra os eritrócitos de ovelhas, confirmando a baixa prevalência deste grupo sanguíneo. Os padrões de reação das amostras são independentes do grupo sanguíneo ABO e do género. O Ac anti-Fs é principalmente, mas não exclusivamente, IgM.

Descritores
Forssman antigen; antibodies; blood group; immunoglobulin; transfusion safety

Abstract

Introduction
Discovered in 1911 by Frederick Forssman, the Forssman (Fs) antigen (Ag) expression varies among species, being rarely present on human red blood cells (RBC). In 1987, three unrelated English families were identified with a phenotype designed *Apae* which was later classified as the 31st blood group: FORS. The antibodies (Ab) anti-Fs have natural occurrence in humans and can interfere in transfusion and transplantation once the Ag is present on the surface of RBC, body fluids and organs.

Goals
The main goal of this research was to evaluate the prevalence of anti-Fs Ab and clarify its impact on transfusion medicine by classifying the type of immunoglobulin (Ig) involved.

Materials and Methods
In this study, standard tube technique was used to evaluate the presence of Ab anti-Fs in plasma samples from a Portuguese donor population and classify the immunoglobulin involved. It was used a 3-5% RBC sheep suspension with a positive expression for Fs Ag to perform all the experiments.

Results
From a total of 11877 samples, 117 (0,99%) showed weak reactions (between 0 and 1 on a scale from 0 to 4) when in contact with the Fs Ag present in the sheep RBC suspension. These samples would be further studied for the search of the mutation in the *GBGT1* gene responsible for the expression of Fs synthase. From the 192 samples studied (50 from each blood type, except AB – the rarest) to classify the Ab involved, 52% revealed to be only IgM, being the remaining samples a mixture between IgG and IgM.

Conclusion
In the population studied, only a few samples (<1%) revealed a negative reaction against the sheep RBC, thus confirming the low prevalence of this blood group. The reaction patterns in the samples are independent of the ABO blood group and gender. The Ab against Fs is mainly, but not exclusively, IgM.

Keywords
Forssman antigen; antibodies; blood group; immunoglobulin; transfusion safety

Introduction

The Forssman (Fs) antigen (Ag) was identified by Frederick Forssman in 1911. This discovery was made by injecting rabbits with a suspension of kidney tissue from guinea pig or horse, and these rabbits were able to produce antibodies (Ab) that hemolyzed sheep red blood cells (RBC) in the presence of the complement proteins. In 1907, Frouin had already mentioned this Ag but only Forssman discoveries led to its recognition [1-3].

This heterophilic Ag is present in a variety of species which were categorized as Fs-positive or Fs-negative, depending on the presence or absence of the Ag, respectively [2-4]. Species as guinea pig, hamster, mouse, cat, dog, horse, turtle, and carp show this Ag in most tissues. Others, like sheep, have the activity of Fs synthase restricted to RBC. Nevertheless, in species like chicken, the presence of the Fs Ag is observed in both erythrocytes and tissues. However, there are also species where Fs synthase is inactive and were considered Fs-negative, not expressing Fs Ag, like goose, pigeon, frog, rabbit, rats, cow and primates including humans [1-3,5-8].

In 1987, Stamps *et al.*, during routine manual grouping, detected for the first time the Fs Ag in humans, initially designated as *Apae*, a subgroup of blood group A. This phenotype was found in three unrelated white English families, although the pattern of reactions was unlike of any group A variant described previously. [9] Later, Svensson and colleagues demonstrated that some anti-A reagents were shown to be nonreactive and this caused some controversy [5,9,10].

However, in analogy with histo-blood group A, Fs Ag is synthesized by Fs-synthase (globoside 3-α-N-acetyl-D-galactosaminyltransferase), a homologous enzyme of ABO transferase and can be used by pathogens as a host receptor leading to transfusion, transplantation and biologic significance [5,10,11].

Although the protein is inactive in most humans, some individuals show Fs Ag which derivate from a genetic polymorphism in the gene GBGT1 – located on chromosome 9 (9q34) - which codify the glycosyltransferase responsible for Fs expression. Svensson *et al.* reported that the first verified and structurally confirmed expression of the Fs Ag on human RBC were individuals with the Apae phenotype encoded an arginine to glutamine change at position 296 which reactivates the human Fs synthase. Consistently, all primates have arginine at position 296 in the enzyme whilst Fs-positive animals have glutamine [1,5,12].

Even though this discovery has many years, only in 2012 the International Society of Blood Transfusion recognized the Fs as the 31st blood group system and, comprising a single Ag with low-prevalence, fulfilling all the requirements by being independent of other blood groups Ag, heritable, expressed on RBC and originates the naturally-occurring of Ab against it [1,5,12,13].

Since most humans do not express the Fs Ag on their RBC surface, the occurrence of expression of Ab anti-Fs is very common and these may play a significant role in binding complement and could cause intravascular lysis of transfused Fs-positive erythrocytes *in vivo* as it caused *in vitro* [5]. However, the expression of this Ag can also be present in body fluids and organs, Yamamoto et al. conclude that the rejection in transplantation through Fs Ab is conditioned by Fs Ag expression on tissue[2], so these Ab anti-Fs may constitute barriers and have repercussions in transfusion medicine, organ transplantation [5,8,14] and even during pregnancies they might be involved in prenatal hemolytic disease [6,15].

Taking into account the above mentioned, we aimed to estimate the prevalence of the Fs Ag and respective Ab in plasma samples from a Portuguese patient and donor population from central region of Portugal, and to clarify the class of immunoglobulin (Ig) produced and its impact on transfusion medicine.

Materials and Methods

Screening of donor plasma samples

During the period of January 1st 2018 and June 30th 2019, the donor samples from the Blood Bank of Coimbra Hospital and University Center were collected for Fs Ab and Ag screening. A total of 11877 plasma donor samples were screened for anti-Fs Ab.

The collected samples were constituted by total blood (6mL) from healthy blood donors and were previously collected by venipuncture into a tripotassium ethylenediaminenetetraacetic acid (EDTA K3) tube. Once at the laboratory, the tubes were centrifuged at 4000g, for six minutes at 22ºC, in the Heraeus Magafuge 16R Centrifuge (Thermo Fisher Scientific, Osterode, Germany), to separate plasma from the cells. Plasma was then transferred into two secondary microtubes properly identified: one for screening and another to be saved at -80ºC as a backup.

For each sample, two drops of plasma were mixed with one drop of 3-5% sheep RBC suspension with Fs Ag expression [16,17] by standard tube technique and investigated for agglutination after one hour of incubation at three different conditions: saline at room temperature (S RT), saline at 37ºC (S 37ºC) and Anti Human Globulin (AHG) both spectrum (IgG+C3d) (one tube for each condition). A plasma sample from an Apae individual was used as a negative control, and monoclonal anti-Fs Ab produced by a mouse lymphoma cell line (M1/22.25.8HL cell line supernatant) in contact with one drop of 3-5% sheep RBC suspension was used for positive control. Plasma samples with weak or none reaction with sheep RBC suspension were further investigated for the Fs Ag presence using gel card haemagglutination, mixing two drops of anti-Fs primary Ab and one drop of 3-5% from their own RBC suspension again for the three different conditions: S RT, S 37ºC and AHG. The cards were incubated for 15 minutes at room temperature (S RT) or 37ºC (S 37ºC and AHG) at the ID-Incubator 37 SI (DiaMed, Cressier sur Morat, Switzerland) and after the incubation, were centrifuged at 1000g in the ID-Centrifuge 24S (DiaMed, Cressier sur Morat, Switzerland) and finally, read.

Immunoglobulin class

After being screened for the presence of the Ab anti-Fs, a total of 192 in 11877 plasma samples from a Portuguese donor population were studied using 2-mercaptoethanol (2-ME) (VWR, Geldenaaksebaan, Belgium) to evaluate the Ab class, as described in the American Association Blood Banks book [18,19].

The samples were used from different ABO and Rh groups in a total of 192 samples: 50 samples from group A (25 Rh-positive and 25 Rh negative), 50 samples from group B (25 Rh-positive and 25 Rh negative), 50 samples from group O (25 Rh-positive and 25 Rh-negative) and 42 samples from group AB (25 Rh-positive and 17 Rh-negative). 250 µl of each plasma sample was mixed with 250 µl of NaCl as negative control and another 250 µl of each plasma sample was mixed with 250 µl of 2-ME at 0.1M. After one hour of incubation at 37ºC, 100 µl of each tube was incubated for 30 minutes at three different conditions: S RT, S 37ºC and AHG and then investigated for agglutination by comparing the reaction in the control tube and the reaction in the tube with 2-ME.

Statistical analysis

The statistical data analysis was performed using the program IBM SPSS® Statistics v.23 (National Opinion Research Center, Chicago, USA).

Was used the Mann-Whitney U test to compare the reaction patterns between males and females' donors. Were also applied the Kruskal-Wallis H and the Mann-Whitney U test to compare anti-Fs Ab reaction patterns for tests carried out at S RT, S 37ºC and AHG for each sample according to ABO and Rh blood groups, respectively.

To measure FORS Ab titre on the samples according to the blood group, was used the Chi-Square test. Finally, to compare the expression of FORS Ab according to gender, was used the Chi-Square test. The differences between the groups under study were considered statistically significant when assuming a random error of $p<0,05$, with a confidence level of 95%.

Results

From a total of 11877 donor samples, 95 samples (0,80%) had a weak reaction for the presence of anti-Fs Ab, where 11 samples had reaction 0 and 84 samples reacted to 0 and 1. The remaining 11782 samples were positive for the anti-Fs Ab presence with different strengths of reaction.

From the donor samples tested, 5355 (45,0%) were Group A, 5199 (43,8%) were Group O, 911 (7,7%) were Group B and 412 (3,5%) were AB (Figure 1). Regarding the Rh system, 9849 (82.9%) of the

11877 donor samples were Rh(D) positive, while 2028 (17.1%) were Rh(D) negative. Considering the gender, 5400 samples (45,5%) were from females and 6477(54,5%) from males.

As exposed earlier, the majority of donor samples were positive for the anti-Fs Ab with different strengths of reactions depending on the medium, temperature and also the donor's ABO group, as can be observed in Figure 2. Regarding the reaction strength between the blood groups, the differences observed in the three different conditions were statistically significant: SRT ($p<0.001$), S37 ($p=0.001$) and AHG ($p=0.002$).

Also, the reaction strength comparison between the genders, comparing the three different conditions, presented statistically significant differences ($p<0.001$) (Figure 3). The female group had stronger reaction patterns than the male group with statistically significant differences ($p<0.001$). Considering the donors' ABO system, were found statistically significant differences ($p<0.05$) between genders for all blood groups, except for the AB.
By studying the class of Ig present, regarding the ABO system, for blood group A, 31 (62%) samples were IgM, 1 (2%) was IgG and 18 (36%) were IgG+IgM; for blood group B, 12 (24%) samples were IgM, 1 (2%) was IgG and 37 (74%) were IgG+IgM; for blood group O, 31 (62%) samples were IgM, 1 (2%) was IgG and 18 (36%) were IgG+IgM and for blood group AB, 26 (62%) samples were IgM, 3 (7%) were IgG and 13 (31%) were IgG+IgM (Table I). Concerning the Rh system, in a total of 100 positive samples, 54 (54%) were IgM, 4 (4%) were IgG and 42 (42%) were IgG+IgM; from 92 negative samples 46 (50%) were IgM, 2 (2%) were IgG and 44 (48%) were IgG+IgM.

Discussion

In 2017 the Blood Bank of Coimbra Hospital and University Center registered 18.000 donors with a total of 15.000 blood donations. As suggested by Stephen M Henry and colleagues, we studied a large collection (11877 samples). Also, considering the total number of annual donations, our donor sampling represents a 99% confidence level with a random margin of error of less than 2%.[20].

By evaluating the prevalence of Fs Ag in this Portuguese population sampling, we have evidence that it is a low-prevalence blood group as reported in previous data [3].

Regarding the AB blood group behaviour, once the structure of Fs Ag is similar to the structure present on the surface of the blood group AB RBC N-acetylgalactosamine - GalNAca [1,3], it's the blood group with weaker reactions.

When we analysed the samples to classify the Ig class involved, we had only 3848 donor samples collected, so we could not manage 25 samples from the AB negative once it is the rarest blood group in Portugal [21]. However, the results revealed that almost all samples had an IgM component, as expected and showed in previous data [6], leading us to believe in its importance in transfusion medicine. An IgG component was also found which may have a significant role during pregnancies. The blood group B which has natural Ab occurring against the blood group A, is also the blood group that has more IgG Ab against the Fs Ag, perfectly understandable once the Fs Ag has been classified as a subgroup of blood group A. As observed in Figure 2 c) the reaction patterns at AHG also corroborate these results.

As it was already described in previous studies, the blood group system FORS is rare in the population[9]. This information is in agreement with our study, where 95 plasma samples had weak or negative reaction patterns when in contact with Fs Ag, which means that there is a high prevalence of the anti-Fs Ab in the Portuguese donor population [9]. Besides that, there are some studies suggesting that the fact of one sample not having the anti-Fs Ab doesn't mean that the Ag Fs is present: it could be due to the fact that the individuals had a low anti-Fs Ab concentration, not detected by the standard technique used, or because these individuals don't produce this Ab [2]. We found statistically significant differences in the three different conditions, regarding the ABO blood group system: SRT, ($p<0.0001$); S37 ($p= 0.001$); AHG ($p=0.002$). Regarding the different blood groups, there were no differences, which means that reaction 3 was the most observed in all blood groups (Figure 2). According to a study developed by Kijimoto-Ochiai

et al. [15], where it was studied the anti-Fs Ab in the human sera in cancer patients, it was concluded that the reaction is independent of the blood group. Still, according to another study developed by Jesus et al [2], the reaction pattern of the anti-Fs Ab does not seem affected by the ABO blood group system. Relatively to gender, it was also found statistically significant differences in the three different conditions ($p<0.001$). In a total of 6477 male samples, the most common reaction pattern was 3 (Figure 3). The same occurred in the 5400 female samples, where the most common reaction pattern was also 3. These results were verified in the three different conditions: S RT, S 37 and AHG. According to the study of Jesus et al [2], the anti-Fs Ab quantity in plasma is higher in females than in males. These results were not verified in the present study.

Conclusion

The prevalence of anti-Fs Ab in a Portuguese donor sample population is high, corroborating that the Fs system is a rare blood group system. We can conclude that this Ab is mostly IgM or IgG+IgM and rarely IgG independent of the blood group. The negative samples should be further studied namely regarding SNP Arg296Gln or any other mutation. A recent study from Santos et al. demonstrated the presence of Galili pentaosylceramide and a Galili heptaosylceramide on the surface of sheep RBC[22]. This way, it would be highly recommended to perform adsorption before using this protocol and these cells [17] to evaluate the presence of anti-Fs Ab in human plasma samples.

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Figure legend

Figure 1. Distribution of ABO donors' blood group in the donors' samples studied.

Figure 2. Reaction patterns of the AB0 group.
a) Reaction patterns at Saline at Room Temperature.
b) Reaction patterns at Saline at 37ºC.
c) Reaction patterns at Anti Human Globulin.

Figure 3. Distribution of the reaction patterns by the gender.
a) Reaction patterns at Saline at Room Temperature.
b) Reaction patterns at Saline at 37ºC.
c) Reaction patterns at Anti Human Globulin.

Figure 1.

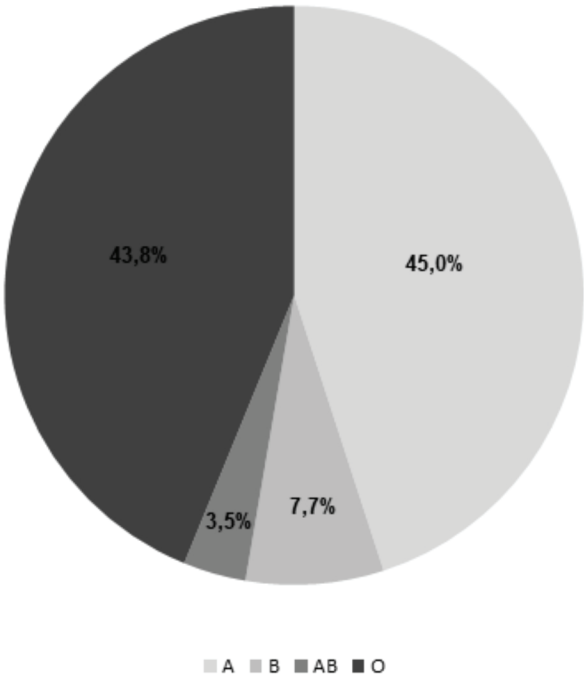
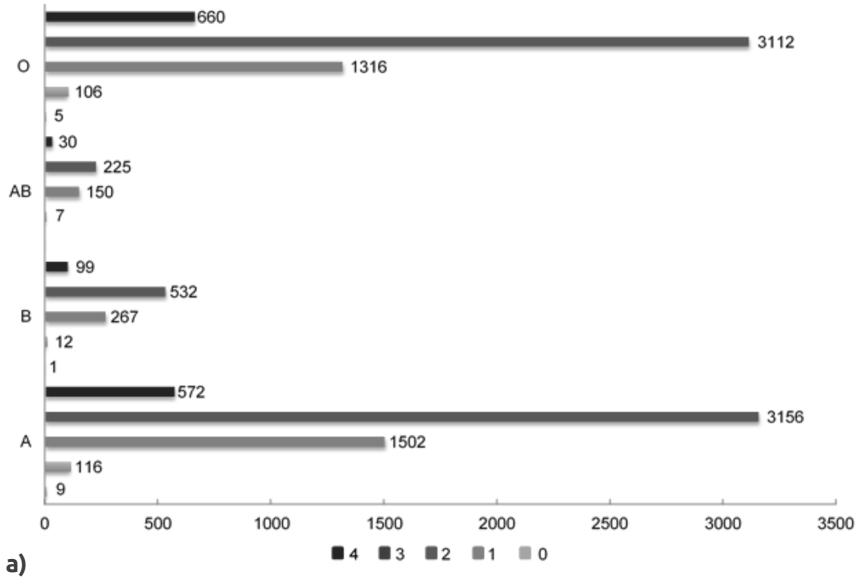


Figure 2.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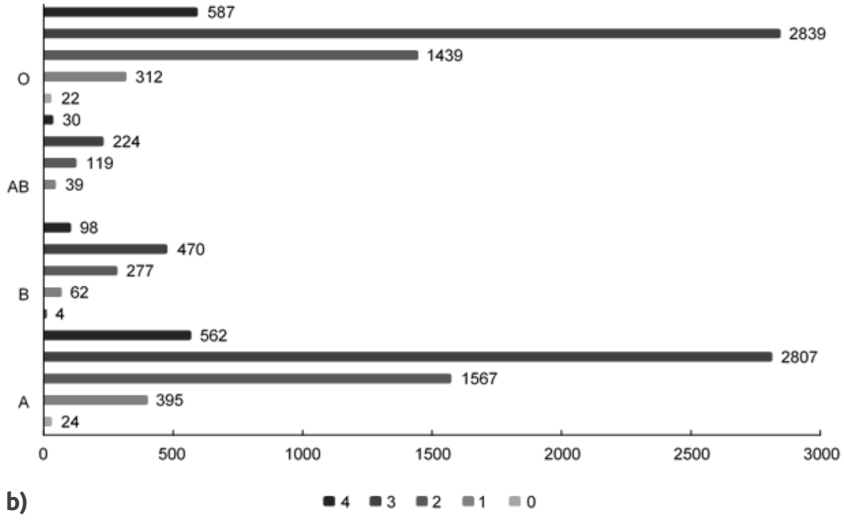
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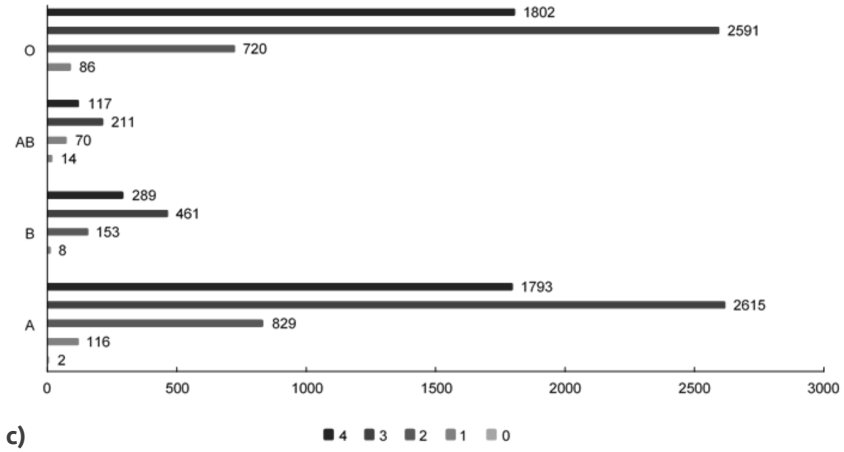
b)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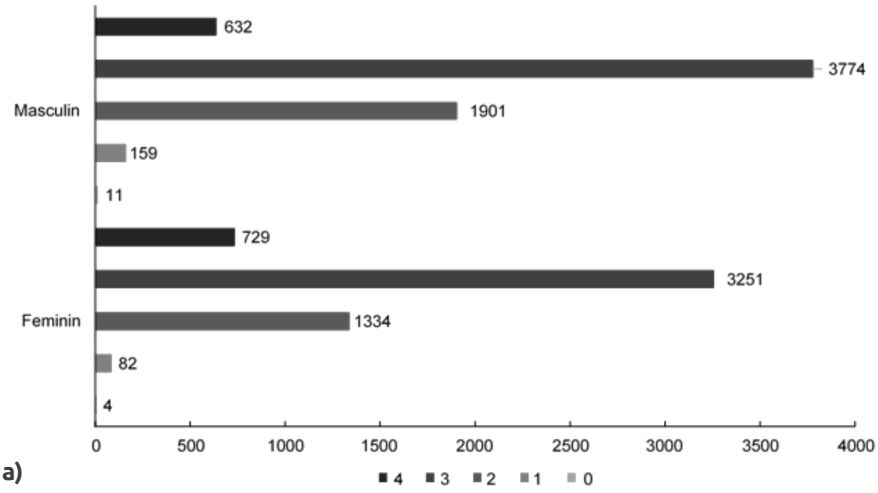
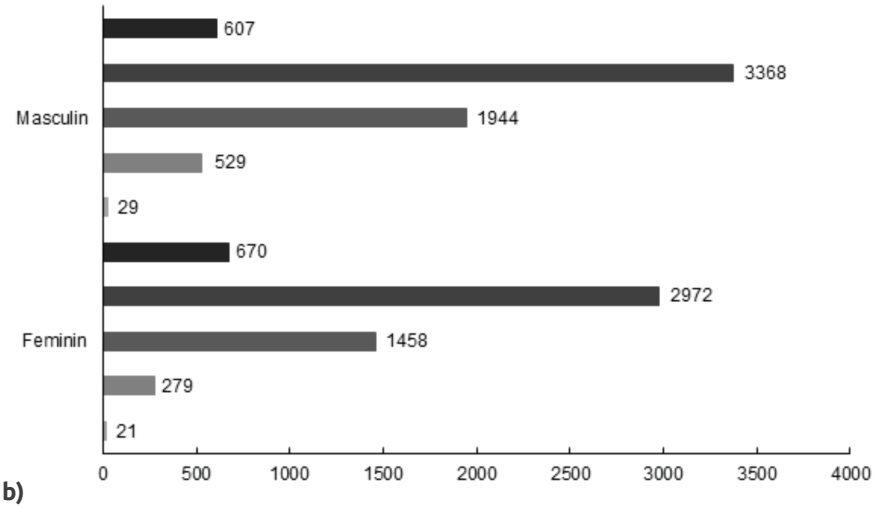
289
461
153
8
A
1793
2615
829
116
2
0 500 1000 1500 2000 2500 3000
4 3 2 1 0

c)

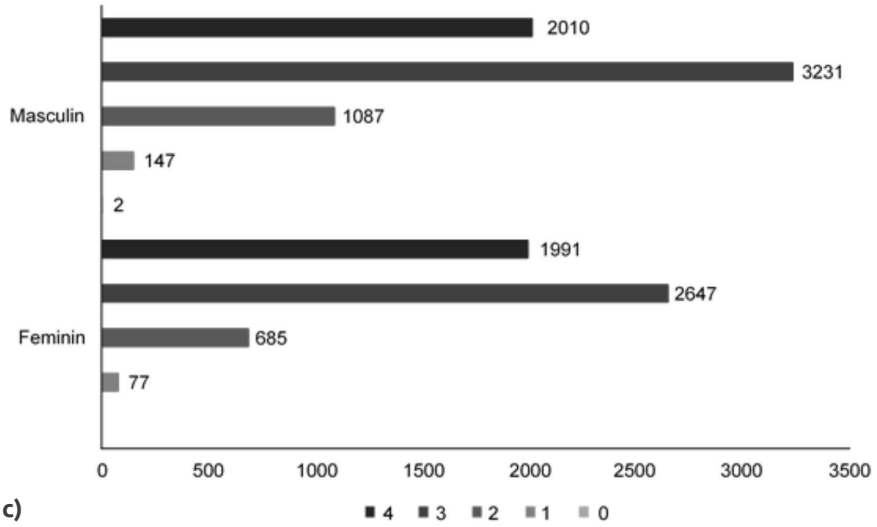
Figure 3.


632
3774
Masculin
1901
159
11
729
3251
Feminin
1334
82
4
0
500
1000
1500
2000
2500
3000
3500
4000
4
3
2
1
0

a)


607
3368
Masculin
1944
529
29
670
2972
Feminin
1458
279
21
0
500
1000
1500
2000
2500
3000
3500
4000

b)


2010
3231
Masculin
1087
147
2
1991
2647
Feminin
685
77
0
500
1000
1500
2000
2500
3000
3500
4
3
2
1
0

c)