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Analysis of gene expression profile for gender in human blood samples

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ABSTRACT: Human peripheral blood is a useful material for biomedical research, since it can be used to investigate responses to treatment and predict diseases. However, various biological and technological factors produce a large degree of variation in blood of gene expression profiles. Our study was based on gene expression profiling analysis on peripheral blood of 45 healthy volunteers, 21 females and 24 men. The blood cells were concentrated, and the total RNA was isolated for the analysis of gene expression using the Affymetrix Gene Chip technology. The results were obtained by a fluorescent scanner, and the numerical data was analyzed using Bioconductor. Samples were clearly divided by gender through the unsupervised clustering analysis. 40 identified genes, differentiating samples by gender were analyzed according to their biological function and chromosomal location. Most of them are located on the X and Y chromosomes. These results provide new insights into the genetic makeup which distinguishes both sexes probably associated with diseases and sex determination.

KEYWORDS: transcriptome, expression, gender, RNA, peripheral blood.

1 BACKGROUND

Peripheral blood is an important source of RNA for gene expression profiling studies (GEPs), because it can be used: to determine the toxicological or disease-related events that have occurred in inaccessible target tissues, to explore the inter-individual GEP associated with donor sex, age, treatments and health conditions., and to describe the influence of technical procedures in GEPs, such as: the conditions of blood sample storage and protocols for RNA extraction and amplification **[1], [2], [3], [4], [5].**

Important differences between males and females exist at every biological level, from the organism as a whole, to organs and organ systems, to individual cells. At the cell level, a major difference is that female cells have two X chromosomes, whereas male cells have one X and one Y chromosome. These biological differences are complex, and may confer variation between gender related to the risk to suffer diseases, like the infection with Hepatitis virus B and C, mental illness and cancer. Furthermore, gender influences in the response to drugs and therapies **[6].**

According to the information published by Cutolo, *et al*. in 1995 and Verthelyi, D. in 2001 **[7], [8]** "Gender is a major determinant of variation in physiology, morphology, and disease susceptibility in humans".

Taking into account the previous information, we set the goal of exploring at a genomic scale the gene expression profile inter-sex. Based on previous research **[1], [9]** we hypothesize that "The expression in peripheral blood of genes associated to sex is mainly determined by genes linked to sex chromosomes, and in smaller degree by genes linked to autosomes, most of them related to diseases conditions such as: infertility, blood diseases and cancer". The importance of this descriptive study resides in providing new information about the inter-sex gene expression profile, in order to help explaining the causes of illnesses and promoting the development of new drugs and therapies.

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2 MATERIALS AND METHODS

2.1 BLOOD SAMPLES DESCRIPTION

2.5 mL of peripheral blood were obtained from 45 apparently healthy Cuban volunteers from 26 to 59 years old (mean±SD=45.42±8, 08). See demographic summary of sample in **Table 1.**

Table 1: Demography summary of samples

	Female	Male
Total # subjects	21	24
Age (mean/sd)	43.95/9.57	47/6.45
Body Mass Index BMI (mean/sd)	27.08/4.81	26.52/3.31
Spanish descendant (% whites)	47.6	62.5
Africans and Spanish mixed (% mulattoes)	23.8	25
African descendant (% blacks)	28.5	12.5

For the efficient blood preservation, the PaxGene collection tubes were used (PreAnalytiX GmbH. Switzerland). Blood was stored at -20ºC until use. Subsequently, the RNA was purified using PaxGene purification kit (PreAnalytiX GmbH. Switzerland), following the manufacturer's recommendations.

2.2 THE RNA QUALITY CONTROL

The RNA quality control (quantity, purity and integrity) was made using NanoDrop; 1000 Spectrophotometer (Thermo scientific, USA) and the Bioanalyzer Agilent 2100, by the use of the Eukaryote RNA 6000 Nano Chip (Agilent technologies, California, USA). All purified RNA exhibited high quality, with an RNA integrity number higher than 7.

To explore the gender-specific differences in mRNA level expression, we compared gene expression patterns between males and females using a whole-genome HG-U133 Plus 2.0 gene chip with more than 46 000 transcripts (Affymetrix Genechip® System, USA).

2.3 DATA ANALYSIS

Microarrays data analysis preprocessing was performed using the bioconductor packages, limma, SAM, affy and simpleaffy **[10].** (Affymetrix data was normalized using the RMA module. A standard Pearson correlation method was used to perform unsupervised hierarchical clustering **[11].** The Limma module of Biocoductor was used to fit a linear model for each probe and P-values were corrected using Benjamini and Hochberg's method to control the false discovery rate **[12].** Class prediction analysis was conducted using the class prediction tools available in the BRB-ArrayTools v3.8 software **[13].** For Gene ontology and disease annotation were used the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 Bioinformatics Resources 6.7, NIAID/NIH, and The human Malady compendium (MalaCard Version 1.05.183 02, Mar 2014), respectively.

3 RESULTS

Gene expression data, from the 45 Affymetrix gene chip were normalized and all was included in the analysis. An unsupervised hierarchical clustering algorithm was run on the data to ascertain if they are separated into groups based on their gene expression profiles. **Fig. 1a** shows different cluster, demonstrating the predominance of two blocks, which contain the greatest number of individuals separated in females (F) and males (M). Sex-differences in gene expressions were characterized by a genome-wide scale using peripheral blood sample collection, allowing for the detection of expression differences with high statistical power. As noted, when the levels of the expression of 46,000 transcripts are taken into account to perform unsupervised cluster with the 45 individuals, two male (2081 and 2036) and three female individuals (2086, 2049 y 2048) are separated from the rest. This might respond to some common factors that affect the expression levels of transcripts in blood, such as temperature, hormones, drugs, the chemicals and other factors that were no controlled in our study. Furthermore, the **Figure 1b** shows a supervised cluster made with the 40 genes that perfectly distinguishes females from males, suggesting it a gene-expression signature for gender.

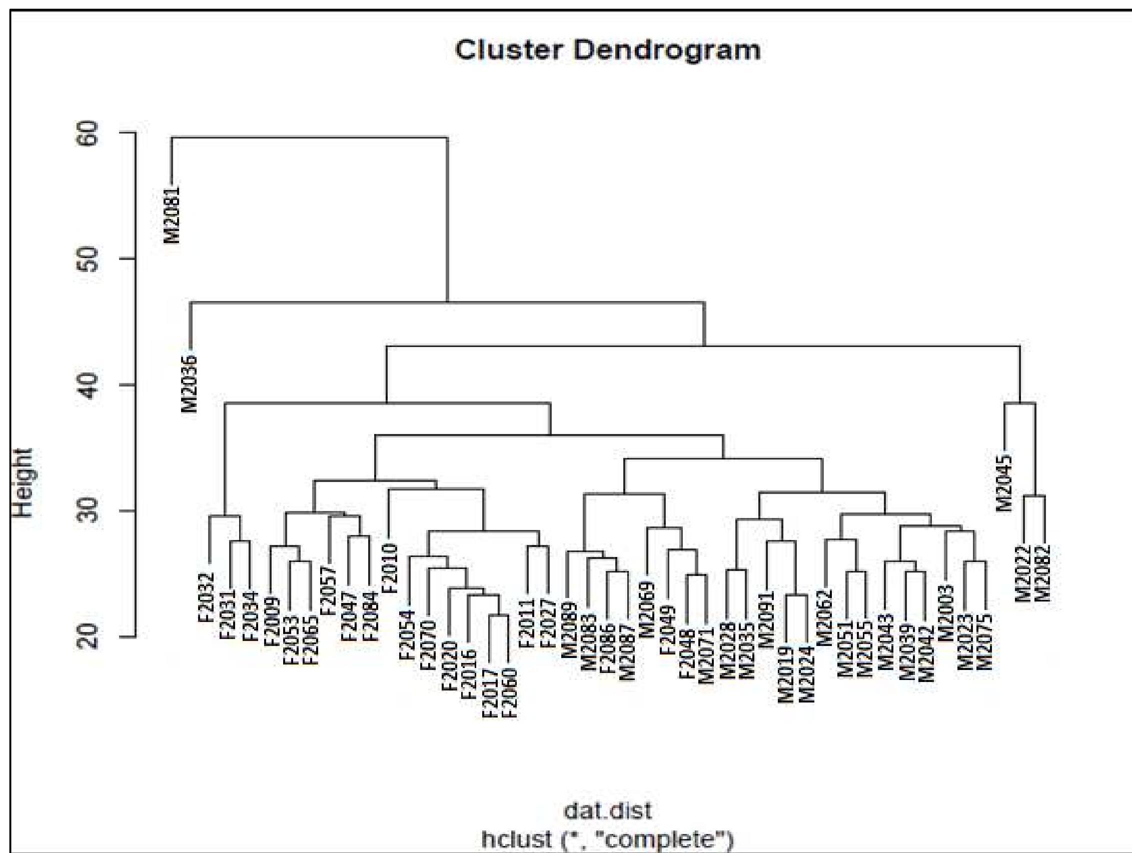

Fig. 1a- Dendrogram of unsupervised hierarchical clustering of Samples, using Pearson's Correlation coefficient as the distance metric. The samples are divided mainly by sex.

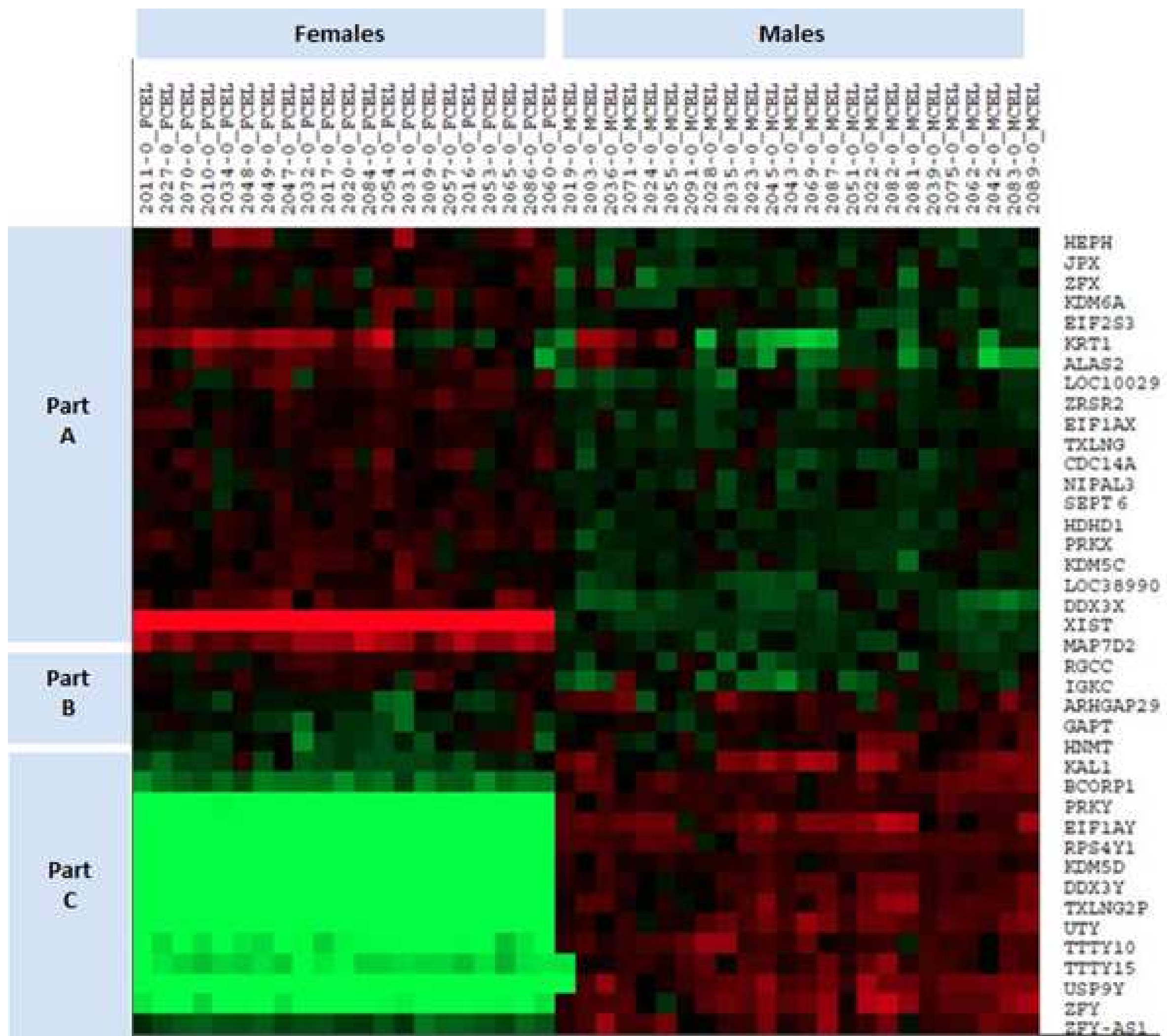

Fig. 1b: Hierarchical supervised cluster of differentially expressed genes associated to sex. Females are represented from the column name 2011 to 2060, and males are represented from the column 2019 to 2089.

40 sex-biased genes were identified with a Fold Change (FC) > 1.4. Among the studied genes, 57.5% showed higher expression in females and 42.5% in males. According to the_17 overexpressed transcripts in males, 76.47% (13 genes) were located on the Y chromosome. On the other hand, of the 23 transcripts overexpressed in females, 73.91% (17 genes) were located on the X-chromosome **(Fig. 2)**. We also identified nine autosomal transcripts with an expression profile different among sexes: KRT1, LOC100291323, RGCC, NIPAL3, GAPT, CDC14A, HNMT, ARHGAP29, and IGKC.

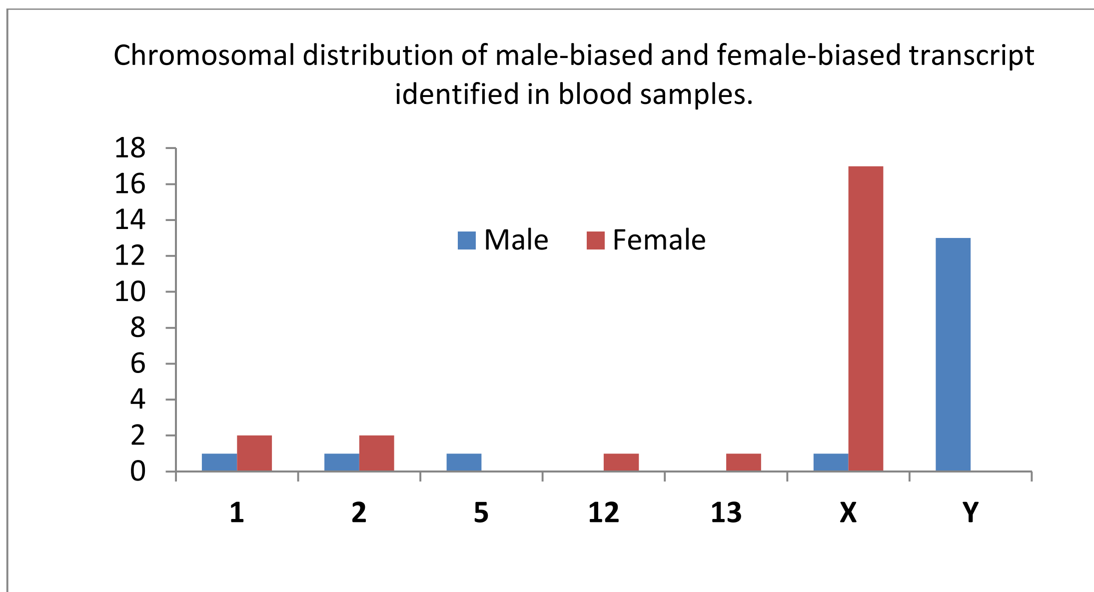

Fig. 2: Chromosomal distribution of overexpressed genes identified in blood samples with male and female-biased.

These differentially expressed genes could represent sex biomarkers. Part A of the cluster, from genes HEPH to MAP7D2 **(Fig. 1b)** represents 45% of the 40 genes with differential expression between gender (all of them are overexpressed in females compared to males). These genes are mainly located on the X chromosome, except for the genes KRT1, CDC14A and NIPAL3 which are located on the autosomes (chromosomes 12 and 1, respectively). Part B of the cluster (from genes RGCC to HNMT) is represented by 22% of all genes. These genes are located on autosomes (chromosomes 1, 2, 5 and 13), and are overexpressed or down-regulated in females in relation to males. Furthermore, the part C of the cluster (from genes KAL1 to ZFY-AS1) is represented by 32% of all the genes, which are mainly located on the Y chromosome and are overexpressed in males in relation to females.

Chromosomal bias was observed with female-biased genes enriched on chrX and male-biased genes enriched on chrY. Analysis of a public information resulted in approximately 77% overlap with our findings **[1], [5], [6], [9], [14], [15], [16].**

Table 2 shows genes with changes in expression greater than 2-fold, and **Table 3** shows genes with changes in expression greater than 1-fold.

Table 2: Genes with a FC>2 of differential expression between sexes.

	Gene symbol	NM	Gene name	Fold change	adj.P.Val	Chr
1	XIST	NR_001564.2	X (inactive)-specific transcript (non-protein coding)	5114,03	7,15e-55	X
2	MAP7D2	NM_152780.3	MAP7 domain containing 2	6,38	2,18e-18	X
3	DDX3X	NM_024005.1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	3,27	5,86e-10	X
4	ALAS2	NM_000032.4	Aminolevulinate, delta-, synthase 2	2,5	3,77e-2	X
6	HEPH	NM_014799.2	Hephaestin	2,16	6,56e-5	X
5	KAL1	NM_000216.2	Kallmann syndrome 1 sequence	-4,16	5,85e-09	X
7	RPS4Y1	NM_001008.3	Ribosomal protein S4, y-linked 1	-2685,75	1,4e-52	Y
8	KDM5D	NM_004653.4	Lysine (k)-specific dease 5D	-3068	2,68e-52	Y
9	DDX3Y	NM_004660.3	Dead(asp-glu-ala-asp) box	-947,99	2,36e-46	Y
10	TXLNG2P	NM_001005852.2	Taxilin gamma 2, pseudogene	-617,72	1,18e-42	Y
11	EIF1AY	NM_004681.2	Eukaryotic translation initiation factor 1A, y-linked	-793,33	3,74e-40	Y
12	PRKY	NM_002760.3	Protein kinase, y-linked	-44,52	5,86e-37	Y
13	UTY	NM_182659.1	Ubiquitously transcribed tetratricopeptide repeat gene, Y-linked	-144,4	1,73e-36	Y
14	TTTY10	Non-protein coding	Testis-specific transcript, Y-linked 10	-33,02	8,26e-30	Y
15	ZFY	NM_003411.3	Zinc finger protein, y-linked	-50,68	1,64e-27	Y
16	USP9Y	NM_004654.3	Ubiquitin specific peptidase 9, Y-linked	-353,97	2,65e-23	Y
17	TTTY15	Non-protein coding	Testis-specific transcript,Y-linked 15	-19,81	2,25e-20	Y
18	BCORP1	NM_001173413.1	BCL6 co-repressor-like 2	-6,47	6,45e-21	Y
19	ZFY-AS1	Non-Protein Coding	Zinc finger protein, Y-linked	-5,02	3,77e-15	Y
20	KRT1	NM_006121.3	Keratin 1	3,86	3,66e-2	12

Table 3: Genes with a FC>1 of differential expression between sexes.

	Gene Symbols	NM	Gene Name	Fold change	adj.P.Val	Chr
1	PRKX	NM_005044.4	Protein kinase, X-linked	1,831	1,89e-06	X
2	HDHD1	NM_012080.4	Haloacid dehalogenase-like hydrolase domain containing 1A	1,623	4,60e-06	X
3	EIF1AX	NM_001412.3	Eukaryotic translation initiation factor 1A, X-linked	1,687	2,31e-05	X
4	LOC389906	Hypothetical protein	Similar to Serine/threonine-protein kinase PRKX (Protein kinase PKX1)	1,844	4,18e-05	X
5	JPX	Non-protein coding	JPX transcript, XIST activator (non-protein coding)	1,580	4,94e-4	X
6	KDM6A	NM_021140.2	Lysine (K)-specific demethylase 6A	1,956	6,57e-4	X
7	TXLNG	NM_018360.2	Taxilin gamma	1,453	1,12e-3	X
8	EIF2S3	NM_001415.3	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	1,622	1,17e-3	X
9	ZRSR2	NM_005089.3	Zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2	1,550	3,19e-3	X
10	KDM5C	NM_004187.3	Lysine (K)-specific demethylase 5C	1,617	4,77e-3	X
11	ZFX	NM_003410.3	Zinc finger protein, X-linked	1,793	1,65e-2	X
12	Septin6	NM_145802	Septin 6	1,409	4,32e-2	X
13	LOC100291323	Hypothetical protein	Similar to immunoglobulin kappa constant; similar to Ig kappa chain	1,963	4,74e-3	2
14	RGCC	NM_014059.2	Regulator of cell cycle	1,699	7,56e-3	13
15	NIPAL3	NM_020448	NIPA-like domain containing 3	1,473	1,21e-2	1
16	GAPT	NM_152687.2	GRB2-Binding Adaptor Protein, Transmembrane	-1,652	2,70e-2	5
17	CDC14A	NM_003672	CDC14 cell division cycle 14 homolog A (S. cerevisiae)	1,645	3,66e-2	1
18	ARHGAP29	NM_004815.3	Rho GTPase activating protein 29	-1,839	3,67e-2	1
19	IGKC	Hs.306357.0	Immunoglobulin kappa constant	1,868	3,77e-2	2
20	HNMT	NM_001024074.2	Histamine N-methyltransferase	-1,802	4,32e-2	2

Genes that discriminate between female and male are involved in 22 biological functions, which could contribute to the establishment of sex dimorphism. Some of them have been validated as biomarkers of sex determination, disease development and drug response (**Table 4**).

Table 4: Gene Ontology analysis of 40 transcripts with differential expression between sexes.

	Biological function	Genes
1	Long non coding RNA (Regulatory function in the genome)	XIST, TTTY10, TTTY15, ZFY-AS1, JPX
2	Histones Demethylase (Epigenetic modification)	UTY, KDM5D, KDM6A, KDM5C
3	ATP-dependent RNA helicase (RNA metabolism)	DDX3Y, PRKY, PRKX, DDX3X
4	Transcriptional activators	ZFY, ZFY-AS1, ZFX
5	Eukaryotic translation initiation factor	EIF1AY, EIF1AX, EIF2S3
6	Cytokinesis (Cell cycle regulation)	SEP6, CDC14A, RGCC
7	Negatively regulates B-cell proliferation	GAPT, LOC3899, NIPAL3
8	Immune response	LOC100291323, RPS4Y1, IGKC
9	Phosphatase	CDC14A, HDHD1
10	GTPase Activity	ARHGAP29
11	Metal ion binding	ZRSR2
12	Posttranslational modification, protein turnover	ALAS2
13	Peptidase	USP9Y
14	Iron metabolism	HEPH
15	Histidine metabolism	HNMT
16	Cellular morphogenesis	PRKX
17	Kinases regulation	KRT1
18	Protein biosynthesis	RPS4Y1
19	X chromosome inactivation	XIST
20	Cell migration during embryogenesis	KAL1
21	Transcription regulation	TXLNG
22	Unknown function	BCORP1, MAP7D2, TXLNG2P

With the aim of linking the differentially expressed genes among sexes with illnesses, we searched for the available information and found that these genes are related to nine conditions, being cancer the most common disease representing 17 genes associated to it (**Table 5**). These associations are based on differences between healthy and unhealthy people with regard to genetic polymorphism and gene expression.

Table 5: Genes associated with sexes represent potential disease biomarkers and future drug targets.

		Genes	References
1	Cancer	RGCC, CDC14A, Septin6, GAPT, KDM5D, DDX3X, ZFX, JPX, XIST, UTY, DDX3Y, EIF1AY, PRKY, ZFY, TTTY15, TTTY10, RPS4Y1, BCORP1.	[17], [18], [19], [20]
2	Infertility	KAL1, ZFY, USP9Y, ZFY-AS1, DDX3Y, EIF1AY, KDM5D.	[21], [22], [23]
3	Psychiatric and neurodegenerative disorders	EPH, KDM6A, EIF2S3, KDM5C, MAP7D2, TXLNG, BCORP1, TXLNG2P, NIPAL3.	[24], [25]
4	Heart Failure	USP9Y, DDX3Y, EIF1AY, RPS4Y1.	[26], [27]
5	Blood diseases	ALAS2, ZRSR2, XIST, HEPH.	[28]
6	Viral diseases	DDX3X, EIF2S3.	[21]
8	Kidney disease	PRK.	[29]
9	Hyperkeratosis, dermatitis and asthma susceptibility	KRT1, HNMT.	[30], [31], [32]
10	Ichthyosis	HDHD1.	[33]

The most represented disease among the 40 sex-linked genes obtained in this study, is cancer. There is a majority representation of genes associated with cancer in sex chromosomes and autosomes, showing the molecular complexity and the high genetic component of the disease with the highest worldwide incidence (**Fig. 3**).

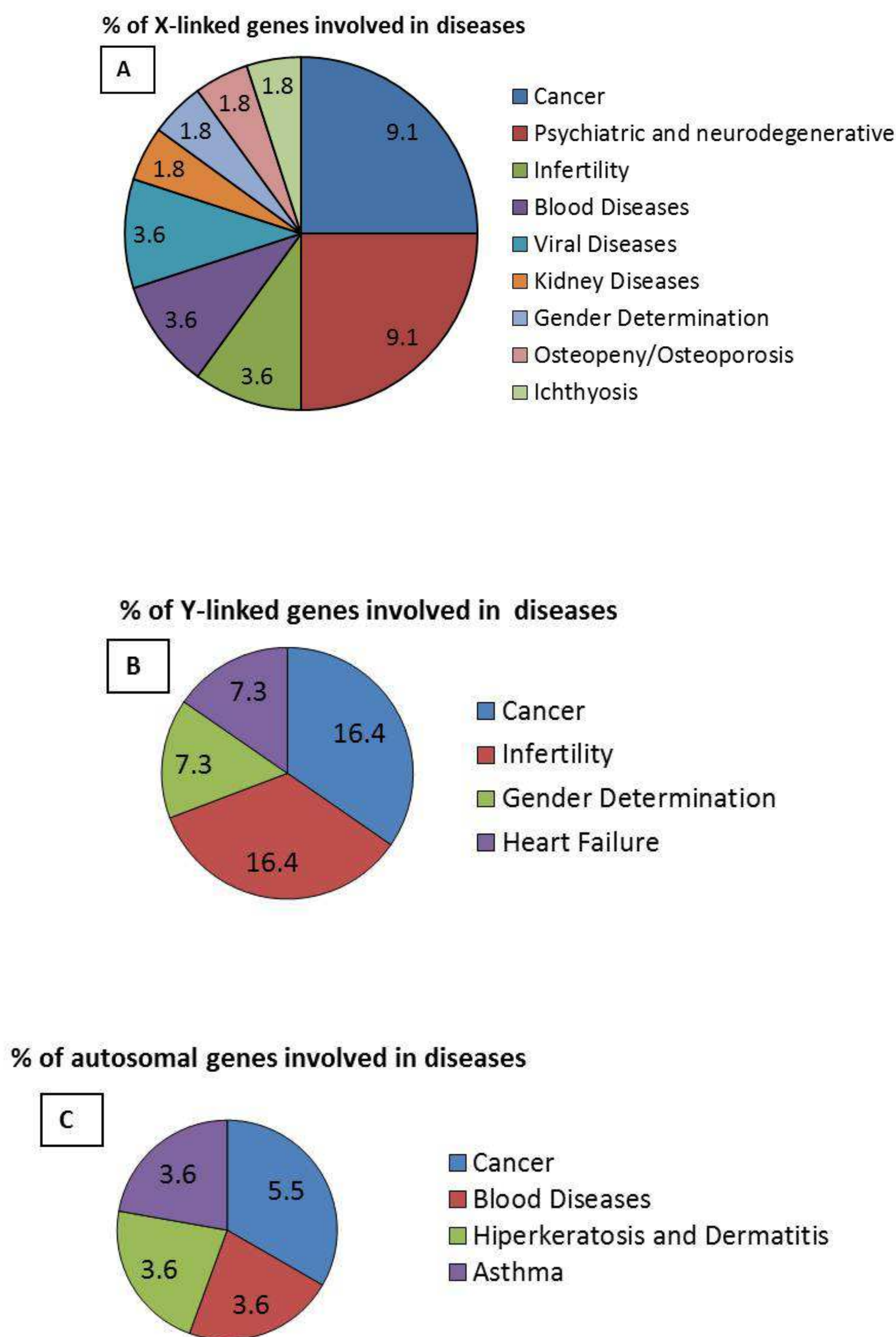

Fig. 3: Sexual chromosomes and diseases representation of the 40 genes with differential expression between sexes. A: represent the % of X-linked genes associated to diseases, with a predominance of genes involved in cancer, neurodegenerative and psychiatric diseases. B: represent the % of Y-linked genes associated to diseases, with a predominance of genes involved in cancer and infertility. C: represent the % of genes in autosomes associated to diseases, with a predominance of genes involved in cancer.

Age also has a strong influence on gene expression [**34**]. To examine whether the effects of age on gene expression, we separately analyzed the data for two age groups (< 45 years N=22; 11 female and 11 men) and >45 years (N=23; 10 female and 23 men). In these two age groups, we identified a set of 73 genes significant by SAM (data not shown). When this list of

genes was used for age classification of samples, the 1-Nearest Neighbor classifier algorithm was the best method, but the sensitivity and specificity was lower than 60%, suggesting that other effects not related with age and not controlled in our experiment may be stronger.

4 DISCUSSION

Of the total genes with significant differences between the sexes, 45% (18 genes) are located on the X chromosome, 32.5% (13 genes) are located on the Y chromosome and 22.5% (9 genes) on autosomes (**Fig. 2 and Tables 2 and 3**). Genes linked to the X chromosome are the most represented in the analysis of differential expression among sexes. Moreover, unlike the previous genes, all genes located on the Y chromosome have a FC>2 of differential expression rate. As is well known, the genes located on the Y chromosome have a distinctive role in sex determination. Some of the most overexpressed genes on the Y chromosome are RPS4Y1, KDM5D, and DDX3Y, which are involved in various biological functions as: eukaryotic translation initiation, epigenetic modification, cell growth, spermatogenesis, RNA metabolism and protein biosynthesis **[35], [36], [37].**

Furthermore, among the overexpressed genes located on X chromosome with the highest Fold Change is the XIST, which is a spliced and polyadenylated “long non-coding RNAs” (lncRNA) typically expressed in all female somatic cells and involved in the initiation of XCI (X chromosome inactivation) in the female cells **[38].** The expression and function of XIST is controlled by other lncRNAs such as JPX (X inactive specific transcript activator), which is described in **Table 3**. A recent work demonstrated the loss of XIST results on X chromosome reactivation and can produce hematopoietic cancers in mice **[20],** showing, in this sense, a direct link between the X chromosome and cancer.

In our transcriptome analysis, the most represented transcripts with expression differences among sexes are non-coding RNA (**Table 4**). The data suggests a possible role of large non-coding RNAs in the epigenetic modification of sex-linked gene expression. It has been suggested by other authors, that gene expression on sex chromosomes can influence the autosomic genes expression, which in turn impact on sex dimorphism observed in human **[39].** This fact could help to explain the inter-sex differences on the autosomic gene expression, being the epigenetics regulation one of the mechanisms involved in the different gene expression profile between sexes. LncRNAs (long non-coding RNAs) have emerged as key molecular players in the regulation of gene expression in different biological processes. Their involvement in epigenetic processes includes the recruitment of histone-modifying enzymes and DNA methyltransferases, leading to the establishment of chromatin conformation patterns that ultimately result in the fine control of genes **[40].**

Among the overexpressed transcripts in males, we found four genes DDX3Y, EIF1AY, PRKY and ZFY which has a functionally overexpressed and interchangeable counterpart on the X-chromosome (genes found on human X and Y chromosomes, in regions that do not recombine during male meiosis. An analysis of nucleotide sequence suggests that these X-Y gene pairs encode similar but non-identical proteins) **[41].** Interestingly in females, the genes DDX3X, EIF1AX, PRKX and ZFX escapes to X inactivation, which would suggest its overexpression in any tissue in females vs. males, due to resultant higher genetic load in females **(Tables 1 and 2) [41].**

It is noteworthy that the KAL 1 gene, which is located on the X chromosome and, according to Franco et al in 1991 [**42],** [**43**] partially escapes to X inactivation, has a lower fold change in females than in males (**Table 1**). But, with the development of new genomic technologies such as microarrays (gene chip) and Hiseq, it has been observed that Kal 1 gene is not found in the list of 114 genes that escape to X-inactivation **[41].** So, the differential expression between female and male may be due to different factors that have an effect on gene expression, such as cellular and hormonal environmental and epigenetic events associated with sexes.

Sex hormones are usually considered the main architects of sex dimorphisms; recent studies have demonstrated that sex chromosomes can also induce sex differences in somatic gene expression in the absence of hormonal differences **[40].** We observed sex differences in the autosomal gene expression which might be related to sex dimorphism. Recent reports on sex differences induced by epigenetic events not hormone-induced, may help to explain the inter sex difference on autosomal genes **[16].**

Diseases with high prevalence in females and males have been reported; in females for example are the autoimmunity diseases, Alzheimer’s disease, and mental illness such as depression and anxiety. In males are cancer, hematologic malignancies, infections, Parkinson disease, autism, alcohol dependence and antisocial personality disorder.

Furthermore, another differences between gender are the clinical symptom presentation (age of disease presentation and evolution) and the response to drugs and therapies **[44], [45], [46].** In this regard, we emphasize that in the female group, there is a prevalence of 9.1% of genes with differential expression inter-sex, which are related to the condition of

psychiatric and neurodegenerative diseases **(Fig. 3).** This result is consistent with the information available about the prevalence of diseases in females in relation to males **[47].**

On the other hand, there is predominance (16.4%) of differential expression of genes related to cancer on the male group, being observed in our results correspondence with the existing information about the association of diseases and sex.

The results show a group of five genes with extreme differential expression between sexes (XIST, RPS4Y1, KDM5D, DDX3Y, and EIF1AY). These genes can be studied and validated as molecular sex biomarker for application in areas such as health and sport. These genes are also involved in the human reproduction, defining the individual fertility, and also they are involved on sex determination during embryonic life and possibly during adult life **[48].**

In this whole blood transcriptome, about 0.1% of genes from the genome changed their expression differentially upon sexes. This is a very low percentage of genes to justify sex dimorphism and all its influence on health and drug response. In addition, other factors may profoundly contribute to sex differences, including: 1) Genetic Factors: genetic polymorphism, and mutations. 2) Epigenetic events: posttranscriptional, posttranslational and environmental and life style factors. 3) Sex hormones: estrogens level s differences. 4) Sex dimorphism: psychological and behavioral differentiation and brain structure and neurochemical **[46], [47], [48], [49], [50]**.

Blood cells, as well as, other tissues show differences in gene expression between sexes. For example, it has been known for decades that the mammalian liver is a sexually dimorphic organ, exhibiting major sex differences in the profile of steroid and drug metabolism **[50], [51], [52].** In humans; it has been observed that there is, in female, a predominant expression of CYP3A4, which is the most important P450 catalyst of drug metabolism in human liver **[52]**. The sexually dimorphic expression of P450s and other liver-expressed genes is regulated by the temporal pattern of plasma growth hormone (GH) released by the pituitary gland, which shows significant sex differences **[52].** The sex-associated differences in the anatomy, physiology and pathophysiology of the human cornea have been also reported. Researchers have identified significant sex-related differences in the diameter, curvature, thickness, and sensitivity and wetting time of the cornea, the mitotic rate of corneal epithelial cells, the density of corneal endothelial cells, and the survival rate of corneal grafts **[53], [54], [55].**

Just like transcriptomic studies examining gene expression profile at the mRNA level, studies comparing the proteome and serum metabolite concentrations of males and females, also found remarkable sex differences. Thus, sex dimorphism most commonly examined at the gene expression levels appear to be present also at the metabolomic and proteomic level **[56]**.

5 CONCLUSIONS

In peripheral blood, the expression of genes associated to sex is mainly determined by genes linked to sex chromosomes (77%), and in smaller degree by genes linked to autosomes (23%). Most of them are related to disease conditions and could contribute to explain the anatomical, behavioral and chemical environmental differences between male and female**.**

All genes located on the Y chromosome have a >2 Fold Change and they are related with infertility and cancer. Genes, located on the X chromosome and autosomes, are related with cancer, psychiatric and neurodegenerative diseases.

This study offer new molecular insights into gender-specific differences providing new genes that could be evaluated as biomarkers of sex and diseases. Our results agree with previous researches showing that female transcriptome is different from the male transcriptome, in which most of differentially expressed genes are located on sex chromosomes**.**

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