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Review

Preclinical models in the study of sex differences

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The biology of sex differences deals with the study of the disparities between females and males and the related biological mechanisms. Gender medicine focuses on the impact of gender and sex on human physiology, pathophysiology and clinical features of diseases that are common to women and men. The term gender refers to a complex interrelation and integration of sex – as a biological and functional determinant – and psychological and cultural behaviours (due to ethnical, social or religious background). The attention to the impact of gender differences on the pathophysiology and, therefore, on the clinical management of the most common diseases, such as cardiovascular diseases (CVD), neurodegenerative disorders, immune and autoimmune diseases as well as several tumours, is in fact often neglected. Hence, studies covering different fields of investigation and including sex differences in the pathogenesis, in diagnostic and prognostic criteria as well as in response to therapy appear mandatory. However, prerequisites for this development are preclinical studies, including *in vitro* and *in vivo* approaches. They represent the first step in the development of a drug or in the comprehension of the pathogenetic mechanisms of diseases, in turn a necessary step for the development of new or more appropriate therapeutic strategies. However, sex differences are still poorly considered and the great majority of preclinical studies do not take into account the relevance of such disparities. In this review, we describe the state of the art of these studies and provide some paradigmatic examples of key fields of investigation, such as oncology, neurology and CVD, where preclinical models should be improved.

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Background

The study of the biology of sex differences as well as the development of gender medicine (GM) is a milestone in the advancement of our knowledge in the different fields of biomedical sciences. In fact, among these fields of investigation are all the research efforts heading for the improvement of our knowledge as concerns the pathogenetic mechanisms of human diseases and the appropriateness of medical interventions in clinical practice. It means that the development or progress of cures could take advantage from the information of sex and gender disparities in order to ascertain and develop differential interventions between women and men. Since the number of human pathologies displaying a significant sex/gender disparity is rapidly growing (Table 1), the needs for investigations specifically devoted to point out the mechanisms underlying sex or gender differences appear mandatory, and the number of works published in the field underscores this assumption.

Human pathological conditions that have been demonstrated to display a gender disparity are really impressive: transmissible and non-transmissible diseases have been investigated in a series of studies aimed at the analysis of the incidence, progression or outcome of very important diseases, such as viral infections, cardiovascular, neurodegenerative, metabolic, respiratory, autoimmune diseases and several forms

Table 1. Human pathologies displaying a significant sex/gender disparity. Some examples of very important diseases, either transmissible or non-transmissible, which show significant gender differences in terms of incidence, progression or symptoms.

Disease	Gender differences			
	Incidence	Progression	Symptoms	Example
Cardiovascular	Yes	Yes	Yes	Infarct
Neurodegenerative	Yes	No	Yes	Alzheimer's
Autoimmune	Yes	Yes	No	Lupus erythematosus
Infectious	Yes	Yes	No	Hepatitis B
Cancer	Yes	Yes	No	Melanoma
Respiratory	Yes	No	No	Chronic obstructive pulmonary disease (COPD)

of cancer [1]. In addition, numerous works underscored sex/gender differences in response to therapy, either for infections, e.g. antiviral therapy, or for non-transmissible diseases such as cancer. Finally, a disparity has been described concerning the adverse effects of pharmacological therapies and for iatrogenic diseases. Being out of the scope of the present work, details of these disparities are simply mentioned here.

A number of works and reviews dealing with these issues can be found in recent literature. For example, the works of the group of Regitz-Zagrosek extensively tackled the cardiovascular matter, at least partially elucidating the key mechanisms underlying the delayed and different features of cardiovascular diseases (CVD) in women [2–4]. In the same vein, other authors started to shed light on the reasons for gender disparity in autoimmune or neurodegenerative diseases [5,6]. However, the biological mechanisms underlying these gender differences are far from being elucidated and surely need further experimental investigations. In particular, to gain this goal, preclinical studies need a reappraisal and a 're-shaping', either as concerns *in vitro* studies or *in vivo* studies in proper animal models [7]. In few words, there is room for all research approaches in this new field called 'GM' as well as in the 'biology of the sex differences' [8]. Of relevance on this matter are the 'Recommendations concerning the new U.S. National Institutes of Health initiative to balance the sex of cells and animals in preclinical research', recently proposed at the Georgetown Consensus Conference and specifically devoted to the implementation and support of preclinical studies taking into account sex in NIH research policy [9].

Gender–sex premise

With regards to the terms sex and gender, they are usually referred to the biological or to the socio-cultural issues, respectively: studies in animals show 'sex differences' whereas 'gender differences' are found in human studies in which environment, self-perception, etc., can be taken into account [10], as also stated by the Canadian Institute of Health Research, http://www.cihr-irsc.gc.ca/e/8673.html. Experimental studies on sex differences are essentially based on three different approaches: research in *in vitro* models, i.e. cultured cells or organ cultures; analyses carried out *ex vivo*, e.g. in cells of peripheral blood and classical preclinical studies carried out in animal models. In particular, as far as cultured cells are concerned, they are usually used for drug screening or mechanistic studies (see below). The cells continuously communicate with each other so that an alteration of one cell can propagate to the others. Hence, this kind of study requires specific analyses on cell–cell as well as cell–substrate interactions. In particular, cell adhesion pathways and communication with other cells can include either sibling cells (homotypic interactions) or other cell types (heterotypic interactions). Two examples: cultured cells normally adhere with each other and to a substrate, so that the propagation of a proliferating or, conversely, of a death signal is very rapid and effective. As concerns the *ex vivo* experimental models, the most paradigmatic example is represented by immune system cells that, belonging to different subpopulations with different roles, need to continuously communicate. This whole scenario can be greatly influenced by the sex. Therefore, even in cytology and cytopathology studies, the sex of the cells (XX or XY) must be taken into account.

In this review, we will recapitulate these issues and discuss the possibility to disclose novel scenarios in the development of diagnostic and therapeutic interventions if and when preclinical studies, e.g. pharmacological and toxicological ones, would take into account both sexes.

Animal studies

Despite decades of research demonstrating sex differences in animal research, many studies are still performed with one sex only [9,11]. The bias towards using only animals of one sex is often based on quite practical reasons: male

mice are larger and thus offer easier targets for attaching electrodes, e.g. in neurological studies, and they do not have oestrous cycles that can complicate pharmacology, toxicological studies in particular. In contrast, females are less aggressive and easier to handle; they are smaller, requiring less weight-administered drug and they are generally less expensive. The importance of such differences varies by strain and by what is under analysis. It is possible, in fact, that males are more responsive in some strains and females in others. For this reason, experimental studies performed by using animals of one sex only might miss some of these differences. For instance, a research team that is mainly testing drugs on male animals might miss beneficial aspects restricted to females contributing to a lack of consistency in experimental results. Also, the costs of not taking sex into account in preclinical studies should be considered: these include failed clinical trials, misdiagnosis and inappropriate therapies for women, as well as omission of fundamental biological principles [12]. On these bases, NIH policy recently encouraged applicants to consider studying both females and males in preclinical biological experiments [13]. This suggestion would avoid research groups assuming no sex difference or ignoring one sex entirely and underscore the relevance of this issue in the development of science. Finally, recent editorial issues asking scientific journals to include both sexes in the experimental analyses or, at least, specify the sex of mice used have recently been published [14]. In few words, the 'instruction to authors' of scientific journals taking into account the sex issue and asking for specific attention to use male and female animals could help in the advancement of experimental medicine.

In vitro studies

In the past century, after the establishment of the first cell lines obtained from human cancers, a series of catalogues of cell lines of different histotype has been made available. More importantly, the widespread use of disposable materials allowing easier, fast and, mainly, reproducible *in vitro* evaluation systems made the use of cultured cells the most suitable investigation tool in the field of experimental medicine [15,16]. In Table 2, the most used and prominent cell lines in experimental studies are listed in order to point out their uselessness as tools for studies taking into account the sex issue. For many years, cultured cells, usually tumour cells, represent the first step for the screening of drugs or toxicants. In addition, a mass of studies aimed at the comprehension of the mechanism of action of drugs or toxicants, as well as the elucidation of genetic or biochemical cell pathways has been carried out in these cell lines. More recently, several non-tumour cells have been cultured *in vitro,* thus providing further information concerning the physiological and pathological features of various cell types, including vessel cells, e.g. fibroblasts, endothelial or vascular smooth muscle cells, as well as other cell types, such as neuronal cells. An important limit of these primary cultures of non-transformed cells in experimental studies is that, unlike tumour cell cultures, they can survive *in vitro* for few subseedings (∼5–7 subseedings). In fact, thereafter, they stop growing and are considered to lose their histospecificity and their 'sex memory' (e.g. their sex-specific chromosomal features are lost, see below). A further series of cultured cell models, such as co-cultures of cells of different types, or cells stably or transiently transfected in order to increase or decrease the expression levels of certain proteins, are also available. These models generally appear irrespective of the original sexual features of these cells.

In this trivial description of the state of the art of cell cultures, several additional pitfalls must be underscored. One example is represented by the reproducibility of the results obtained with the same cell line in different laboratories. Some years ago it was suggested that the same cell line from the same collection could provide dissimilar results in parallel identical experiments performed in different labs. This was due, at least partially, to the genetic instability or to post-transcriptional changes occurring after several *in vitro* subseedings, due to the selection of various clones of the same cell line in different laboratories. In fact, cell lines with an abnormal number of chromosomes or with constitutive overexpression or the lack of growth factor or hormone receptors can usually be found. For these reasons, a growing number of scientific journals require authors to check authentication of the origin and identity of the cells by DNA profiling. Also, for tumour cells freshly isolated from patient tissue or in the case of a new cell line, the DNA profile needs to be cross-checked with the DNA profile of the donor tissue. This could be of great relevance in order to develop sex-specific treatments, as recently suggested for chemotherapy [17,18].

On these bases, it appears clear how difficult it is to conduct cellular physiopathology studies in which the cell functions or dysfunctions, object of the research, may be related to the cell sex by using stabilized cell lines. It means that a comparative analysis of the same cell type should be conducted on male or female freshly isolated cells to point out a possible sex-specific pathway. In few words, to get reproducible and sex-tailored results in basic or translational science, cell models that maintain sexual specificity should be considered. Moreover, in line with the fundamental review paper by Shah et al. [15], it is clearly inappropriate to assume that results from studies conducted on only one sex will apply wholesale to the other.

Table 2. Cell lines of widespread use in experimental studies. The salient features of these cell lines are listed in order to point out their uselessness as tools for studies taking into account sex differences (see text for details). Cells indicated as normal or noncancerous are often virally transformed. This table is not intended to be a comprehensive data set, but rather to highlight the cell lines that are routinely used.

Name	Morphology	Species, sex, age	Origin	Isolation date
HeLa	Epidermoid	Human, female, 31	Adenocarcinoma of the cervix, HPV-18+	1951
HEp-2	Epidermoid	Human, male	Carcinoma of the larynx, HPV+, contaminated with HeLa	1952
CHO-K1	Epithelial	Chinese hamster, female	Ovary	1958
3T3-L1	Fibroblast	Mouse embryo, sex unknown	Fibroblast	1962
Vero	Epithelial	Green monkey, sex unknown	Kidney	1962
Raji	Lymphoblast	Human, male, 11	Burkitt's lymphoma, B-cells EBV+	1963
CEM	Lymphoblast	Human, female	ALL, T-cells, EBV+	1964
HT-29	Epithelial	Human, female, 44	Colorectal adenocarcinoma	1964
MDCK	Epithelial	Dog, sex unknown	Kidney	1966
U-87 MG	Epithelial	Human, male, 44	Glioblastoma	1968
Jurkat	Lymphoblast	Human, male, 14	ALL, T-cells	1970
MCF7	Epithelial	Human, female, 69	Breast cancer	1970
SH-SY5Y	Epithelial	Human, female, 4	Neuroblastoma	1970
A549	Epithelial	Human, male	Lung carcinoma	1972
A-375	Epithelial	Human, female, 54	Malignant melanoma	1973
Saos-2	Epithelial	Human, female, 11	Osteosarcoma	1973
U937	Monocyte	Human, male, 37	Histiocytic lymphoma	1974
K-562	Lymphoblast	Human, female, 53	CML	1975
H9c2	Myoblast	Rat embryo, sex unknown	Heart tissue	1976
PC12	Irregularly shaped cells	Rat, male	Pheochromocytoma	1976
CaCo-2	Epithelial	Human, male	Colon cancer	1977
HL-60	Myeloblastic	Human, female, 36	Acute promyelocytic leukaemia	1977
IEC6	Epithelial	Rat, male	Small intestine	1979
NCI-H292	Epithelial	Human, female, 32	Mucoepidermoid pulmonary carcinoma	1985
PNT1A	Epithelial	Human, male, 35	Prostate	1991
HEP G2	Epithelial	Human, male	Liver cancer	1994
Cor.4U	Cardiomyocyte	Human, female, 26	*In vitro* differentiated stem cells	Recently isolated

Some paradigmatic examples

The sex-tailored approach could be of great importance in the screening and development of new drugs or therapeutic strategies that could take into account the differences between XX and XY cells. Some specific examples come from literature data reporting 'cell-sex' as a key variable in the response to exogenous agents [15–17]. In a series of works, our group underscored the disparity of cells from males (XY) and females (XX) in their response to an exogenous stress [4,19–22]. Primary cultures of cells of different histotypes from different animal species, such as endothelial cells, smooth muscle cells, fibroblasts, neuronal cells from mice, rats or humans provided a number of results that can be summarized as follows: under the same stressing conditions, somewhat mimicking an inflammatory state, cells from males undergo cell death more easily (apoptotic triggering), whereas cells from females, thanks to a potent antioxidant power, counteract the stressing molecules, i.e. reactive oxygen species, and survive better. Some works also refer to the XX and XY cell disparity as a difference in metabolic response. For instance, during starvation conditions, neurons from males more readily decreased mitochondrial respiration undergoing death, whereas neurons from females mobilize fatty acids, accumulate triacylglycerols, form lipid droplets and survive longer [21,23,24]. This represents a key issue of *in vitro* cultured system investigations: the different 'frailty' of XX and XY cells, the former displaying a higher plasticity to environmental stress, that results in a higher rate of survival or proliferation, through the cytoprotection mechanisms of autophagy and senescence, the latter more easily undergoing the programmed cell death pathway [19,21,22,24].

Table 3. The main *ex vivo* systems useful to investigate the pathogenetic mechanisms of diseases.

Cell type	Animal species	Maintaining 'sex memory' (number of passages)	Experimental tools for
Fibroblasts	Human, mouse, rat	~10	CVD, autoimmune
Vascular smooth muscle cells (VSMC)	Human, mouse, rat	~10	CVD, gastroenterology
Keratinocytes	Human	8–10	Dermatology
Endothelial cells	Human (from umbilical cord)	~10	Vascular
Cardiomyocytes	Rat, mouse	~5	CVD
Central nervous system cells (neurons, glia, astrocytes)	Rat, mouse	–	Neurodegeneration
Mouse embryo fibroblasts (MEF)	Mouse	15–20	Cell biology, cell pathology
Freshly isolated cancer cells	Human, mouse	10–15	Experimental oncology
Peripheral blood lymphocytes	Human	–	Immunity, inflammation, experimental oncology
Platelets	Human	–	Haematology, CVD
Red blood cells	Human	–	Haematology, CVD

Ex vivo studies

The term *ex vivo* refers to an experimental model consisting of cells or tissues from an organism kept in an external environment with minimal alteration of 'natural' conditions. *Ex vivo* systems allow us to experiment on freshly isolated cells under more controlled conditions than in *in vivo* models. The analysis of peripheral blood cells certainly represents a great tool for translational studies in various fields of investigation, such as research for diagnostic and prognostic purposes as well as for investigations of the pathogenetic mechanisms of diseases. Although inflammatory, immune and autoimmune diseases can be considered as paradigmatic fields of investigation, a number of haematologic diseases or disorders leading to peripheral blood cell alterations also represents a milestone. It means that platelets or red blood cell alterations have to be considered in this field [25–30]. Even if the main goal of these studies is related to the improvement of diagnostic matters, the implication of these *ex vivo* analyses in the advancement of our knowledge of the pathogenetic mechanisms of diseases in a sex perspective is of great relevance. The main *ex vivo* experimental models have been summarized in Table 3.

Some paradigmatic examples

The first point concerns the use of peripheral blood mononuclear cells (PBMC) or lymphocytes (PBL). A good example dealing with these tools is represented by the study of autoimmune diseases, often associated with disturbances of PBL. These cells, directly taken up from the peripheral blood of patients with autoimmune diseases, such as systemic lupus erythematosus (SLE) or rheumatoid arthritis – displaying a significant gender disparity in their incidence (even 9-11:1 F:M) – can be studied in detail for their integrity and function. However, to date, very few studies have specifically taken into account the gender issue so that only recently some of the possible determinants of gender disparity in these diseases have been discovered [31–34]. In particular, a role for oestrogen has been envisaged in SLE in which the presence of autoantibodies to the oestrogen receptor-α has been observed. These autoantibodies could interfere with T-lymphocyte homoeostasis thus playing a role in disease activity [27]. Several other examples suggest that major sex differences exist in the mechanisms underlying immune modulation of various diseases [35]. A recent study reported that the adaptive immune system could be implicated in the development of hypertension in males but not in females due either to the role of the immune system in the development of high blood pressure in females or to ovarian hormones. In fact, in women, natural menopause is known to result in significant changes in the expression of genes regulating the immune system [36]. Unfortunately, confounding factors can complicate these analyses: (i) lymphocyte activation pathways, (ii) the presence of different lymphocytes subsets and (iii) the interaction among these cell subpopulations. These factors, making data interpretation difficult, impair the advancement of our knowledge and the proposal of conclusive hypotheses. Hence, albeit the literature data on the implication of cell sex on immune system function, as well as gender disparity in immunoregulation, are well documented, research in this area is still at the beginning and needs a strong incentive from funding agencies and scientific journal editors that could be interested to this matter.

As concerns non-nucleated cells taken up by the peripheral blood, i.e. platelets and red blood cells, even these cell types have been suggested to represent useful tools to dissect sex differences in human pathology. In particular, the

usefulness of these cell types as real-time biomarkers, as diagnostic or disease progression determinants has also been proposed in a gender perspective [26,37,38]. A good example is represented by sex variability in platelet aggregation in response to common agonists [30]. For instance, by using an *ex vivo* experimental system, it has been observed that sex can represent 'a determinant of agonist effects on platelet aggregability even in healthy subjects'. The mechanisms underlying this disparity could be related to the relevance of the oestrogen–oestrogen receptor system in platelet homoeostasis (reviewed in [37]).

Here below we will examine the main experimental models used in the study of some important human diseases focusing on sex and gender. Such information comes from research done on the PubMed website using the search strings listed in Supplementary Figures S1 and S2.

Oncology
Experimental models
For ethical reasons, the number of animals used in biomedical experiments has strongly been reduced in the last 20 years. However, this number had to be adequate for the statistical power required to generate robust data. Many preclinical animal models in oncology drug development fail to accurately predict the clinical efficacy of novel anti-cancer agents, mainly due to their inability to reflect the complexity and heterogeneity of human tumours. The most commonly used models for the study of cancer are the human tumour xenografts, humanized mice models, in which immunodeficient mice are engrafted with human cells or tissues, which are considered extremely useful for functional research *in vivo* studies.

Cancer cell lines have widely been used for research purposes and proved to be useful tools in the genetic approach. Results show that their characterization is, in fact, an excellent model for understanding the biological mechanisms involved in cancer [15]. The use of cancer cell lines allowed increased information about the deregulated genes and signalling pathways underlying this disease. Furthermore, cell models represent the first step in the development and testing of anticancer drugs presently used, and in the development of innovative therapies, but also an alternative to transplantable animal tumours in chemotherapeutics testing. In fact, the use of the appropriate *in vitro* model in cancer research is crucial for the investigation of genetic, epigenetic and biochemical pathways, for the study of proliferation deregulation, apoptosis and cancer progression, to define potential molecular markers, and for the screening and characterization of cancer therapeutics. Very recently, the relevance of sexual dimorphism has been discussed in detail as concerns basic science research in the field of oncology [17].

Although gender disparity in the incidence, aggressiveness and disease prognosis have been observed for a variety of cancers, clinical trials and research in animal models are still gender unbalanced [39]. Gender-specific oncology needs to reconstruct a correct equilibrium in order to understand the molecular basis underlying the gender differences in the outcome and response to therapies. The interactive database GenderMedDB [40], which enables the retrieval of pre-selected publications containing sex- and gender-specific analyses, shows that in the field of oncology, sex-related publications have increased in the last 10 years (Figure 1A). In particular, melanoma, bladder, stomach and thyroid cancers are the most represented (Figure 1B).

Here, we explore sex differences in preclinical studies of two important forms of cancer: melanoma, as a solid tumour example, and leukaemia/lymphomas as blood cancers, focusing on the experimental models used.

Melanoma
Metastatic melanoma is one of the fastest growing cancers, causing more than 8650 deaths in 2009 alone, and this number is projected to increase over time [41]. Gender-specific differences in melanoma epidemiology are well established. The probability of developing melanoma during one's lifetime is 1.72% in men and 1.22% in women. In the Netherlands, a large population-based cohort study including 10538 melanoma patients from 1993 to 2004 analysed the gender difference in melanoma survival after adjusting for tumour-related variables (Breslow thickness, histology, tumour site, and metastatic and nodal status). It has been found that the relative excess risk of mortality was 2.70 in males compared with females. The female survival advantage remained after adjusting for multiple confounding variables including tumour thickness. Gamba and colleagues recently analysed data from 26107 individuals, age 15–39 years, from the US National Cancer Institute's Surveillance, Epidemiology and End Results registry. They reported that young men had a 55% lower rate of melanoma survival compared with age-matched young women, and concluded that male sex, within all specific age groups and across all tumour thickness categories, histologic subtypes and anatomic sites, is associated with a disproportionate burden of melanoma deaths [42]. Localized melanomas in women showed a lower risk of metastasis, resulting in a better survival when compared with men, even after first disease progression. In localized melanoma, men generally had worse characteristics at diagnosis, such as older age,
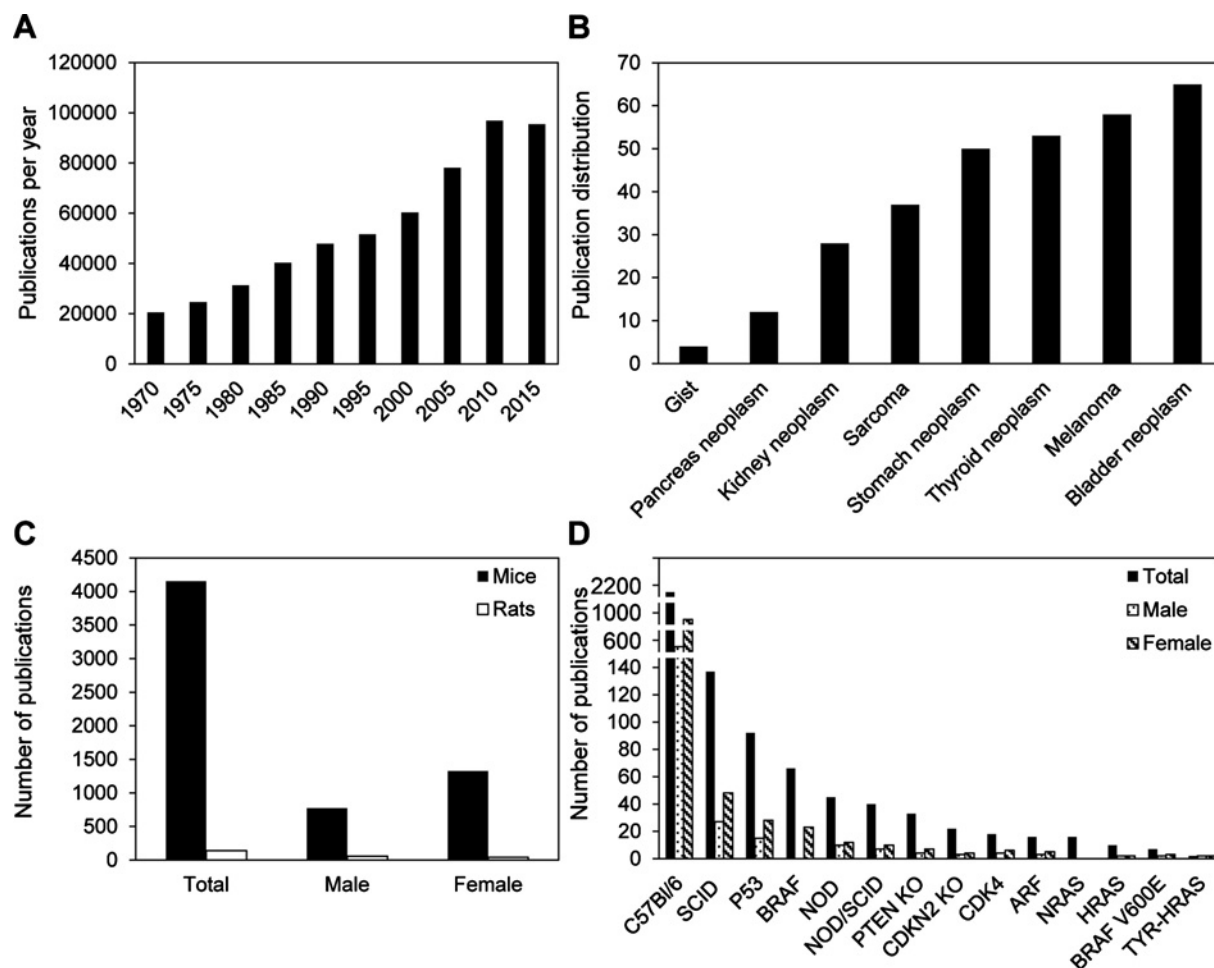
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Figure 1. Animal models for melanoma studies

(**A**) Oncology-related publications per year (data from PubMed.gov). (**B**) Publication distribution within diseases (extrapolated data from GenderMedDB database). (**C**) Number of publications showed in MEDLINE in the last 10 years where scientists report the use of mice or rats and the gender for melanoma studies. (**D**) As a result of the large number of published melanoma models, we can discuss only an arbitrary selection of the currently available models in this review considering the major signalling networks deregulated in melanoma by mutation or other genomic alterations, which include: (i) xeno-transplantation models; (ii) syngeneic transplantation models and (iii) models involving genetically modified animals (mutant BRAF or mutant NRAS), and/or via inactivation of key tumour suppressors, including CDKN2A, PTEN or BRAF V600E that cooperate with PTEN loss to induce metastatic melanoma. The relevance of each particular model depends on how closely it represents the genetic and epigenetic aberrations, histology, physiological effects and metastatic pattern observed in human melanoma.

increased likelihood of having an ulcerated or thicker primary tumour, melanomas more commonly located on the head, neck and trunk, and less often on the extremities. However, even after diagnosis, men continue to have disadvantages compared with women. In various studies, women showed a longer delay before relapse and higher cure rate compared with men [43]. On the basis of these results, we identified, by optimizing the search of the MEDLINE database incorporating Medical Subject Headings (MeSH®) (Supplementary Figure S1), the number of publications related to animal studies performed with male or female mice. As shown in Figure 1(C), female mice are the most used animals for preclinical studies in melanoma. Several mouse melanoma models have been developed and are used: (i) to determine the function of particular proteins in melanoma progression; (ii) to approximate certain biological aspects of human melanomas and (iii) to critically evaluate novel drugs/therapies. In particular, C57Bl/6, immunodeficient mice (SCID, NOD and NOD/SCID) and engineered mice (such as BRAF and p53) strains are the most used (Figure 1D).
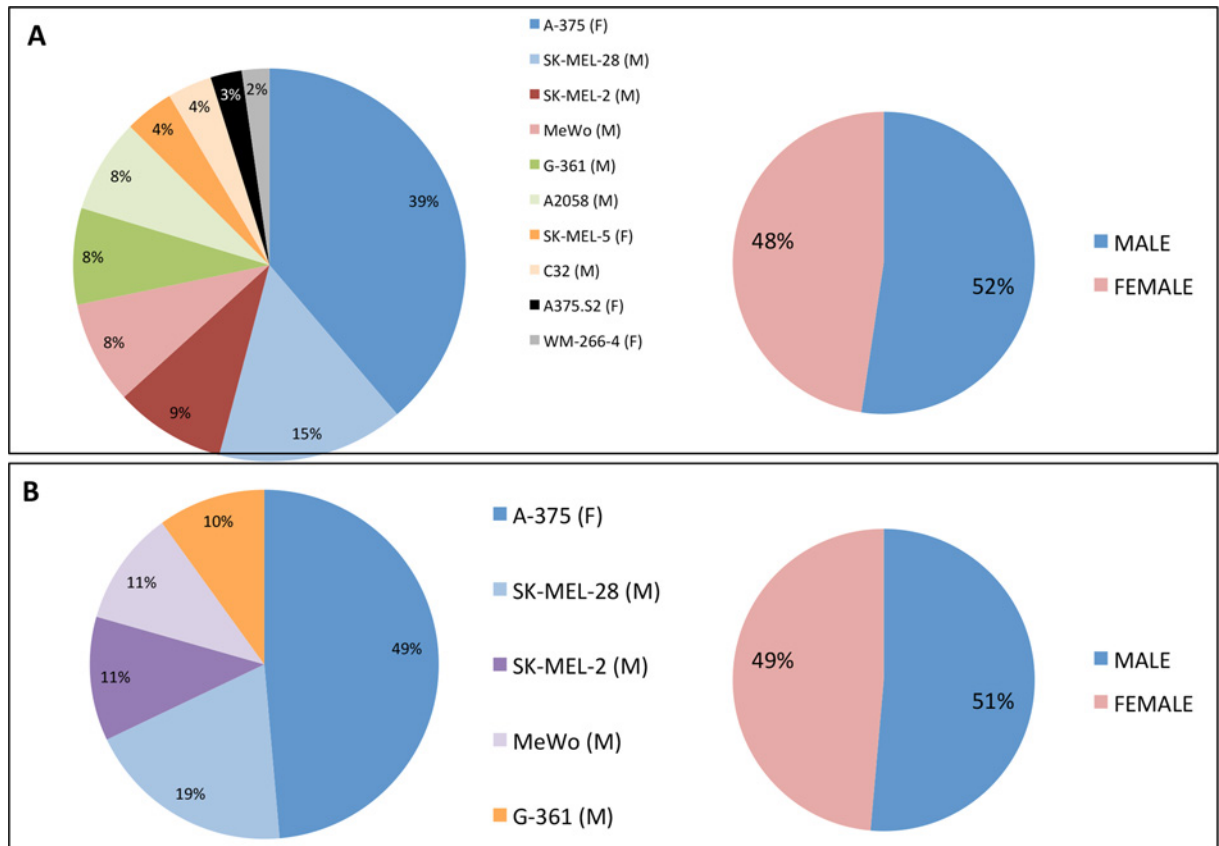
Figure 2. Most used cell lines for melanoma studies

(**A**) Pie chart depicting the relative distribution of the scientific papers in which the 10 most used melanoma cell lines have been used as an *in vitro* model (left panel). These 10 examined cell lines represent 91% of the cell lines used in the literature for *in vitro* studies on melanoma. Pie chart depicting the sex distribution of the 10 most used cell lines in the literature (right panel). (**B**) Pie charts depicting the same analysis of panel A but restricted to the five most used cell lines. These five examined cell lines represent 71% of the cell lines used in the literature for *in vitro* studies on melanoma.

As far as the *in vitro* studies were concerned, we analysed the main cancer cell lines used in the literature through a specific search-string on PubMed (Supplementary Figure S1). In particular, we have analysed 50 cell lines of commercially available melanoma. Within this list we examined the 10 most used cell lines representing 91% of the cell lines used in the literature for *in vitro* studies on melanoma, as shown in Figure 2(A) (left panel). All of these cell lines have been isolated in the 1970s from patients with a mean age of 51 years old (y.o.). A-375 is the most used cell line, being reported in 38% of the total number of selected papers. It was isolated in 1973 from a 54 y.o. female patient, and originates from a primary lesion of a malignant melanoma showing a BRAF mutation [44]. In this top 10 list, cell lines from males and females are equally represented (Figure 2A, right panel). Going to restrict our analysis to the top five most used cell lines (Figure 2B, left panel), we observed that these are employed in 71% of the published papers. In addition, although the percentage of male and female cell lines remains unchanged (Figure 2B, right panel), we can notice that the whole amount of studies using cell lines from females can be attributed to one cell line, the A-375 cell line.

A very interesting point has recently been underlined as concerns genetic differences between men and women by an analysis of mutation spectra in 266 metastatic melanomas. These analyses demonstrate for the first time a gender difference in the mutation burden of cutaneous melanoma [18]. A recent study also found that female patients with melanoma had a significantly higher frequency of tumour-associated, antigen-specific CD4$^+$ T-cells than their male counterparts [45]. In consideration of the relevance of recent advances in the field of molecularly targeted therapy and immunotherapy, these results could open new scenarios also in the field of GM.

Leukaemia

Since sex differences have been observed also for leukaemia, the main features of this disease are first very briefly described here. There are four main types of leukaemia: (i) acute myelogenous leukaemia (AML), a fast-growing form of cancer of the blood and bone marrow, that can occur in children and adults, (ii) acute lymphocytic leukaemia (ALL) mostly occurring in children [46], (iii) chronic myelogenous leukaemia (CML), a form of cancer that affects the bone marrow and blood, that mostly affects adults. It begins in the blood-forming cells of the bone marrow and then, over time, spreads to the blood; (iv) chronic lymphocytic leukaemia (CLL) very rarely seen in children and is most likely to affect people over the age of 55. CLL is a typically slow-growing cancer, which begins in lymphocytes in the bone marrow and extends into the blood.

As reported in the Surveillance, Epidemiology and End Results Program, an authoritative source of information on cancer incidence and survival in the US, the number of new cases of leukaemia was 13.5 per 100000 men and women per year. The number of deaths was 6.9 per 100000 men and women per year (these rates are age-adjusted and based on 2009–2013 cases and deaths). Importantly, the number of new cases is 17.3 per 100000 males compared with 10.5 per 100000 females. Differences in sex-specific incidence and prognosis have long been recognized for CML. In this pathology, male predominance was demonstrated in clinical trials, and the Sokal score for younger patients (<45 years old) identified female sex as a favourable prognostic factor [47]. In the context of B-cell lymphoproliferative disorders, a marked preponderance of men (4:1 male to female ratio) is reported in diseases such as hairy cell leukaemia [48]. Further differences between men and women have referred to sex variation in drug efficacy and toxicity, particularly sex differences in pharmacokinetics [49]. As reported by Catovsky and colleagues, women affected by CLL show a better overall response to treatment than men but greater gastrointestinal toxicity. No good hypotheses have been advanced to explain the observed trend for a better outcome in women. There are three possible factors that may contribute to the better treatment response and longer survival in women: (i) the association with good prognostic factors, (ii) pharmacokinetic differences between the sexes and (iii) the effect of oestrogens [50].

Preclinical studies with animal models provided important insights for the understanding of the molecular determinants of leukaemogenesis also exploring new drugs or combinations [51]. As shown in Figure 3, there are several animal models, mainly female mice, for use in leukaemia research (Figures 3A and 3B). However, the lack of heterogeneity and inter-case variability of animal leukaemia models limits the transferability of the results into the clinical setting.

Concerning the *in vitro* cell models of leukaemia, the 10 most commonly used cell lines (among 60 commercially available), represent the great majority (92%) of the cell lines used in preclinical studies on leukaemia (Figure 4A, left panel). All these cell lines have been isolated from 1964 to 1980, from patients with a mean age of 29 y.o. HL-60 is the most used one, being reported in 36% of the total selected papers. HL-60 was isolated in 1977 from a 36-year-old woman with acute promyelocytic leukaemia at the National Cancer Institute [52]. As clearly depicted in Figure 4(A) (right panel), cell lines from males and females are not equally represented. In fact, in 70% of studies, cell lines from female patients have been used, and in several studies the 'sex' of the analysed cell lines was not mentioned at all. When we restricted our analyses to the first five cell lines used in these studies (Figure 4B, left panel), we observed that they represented 82% of all published papers. Furthermore, the male/female imbalance further increased the disadvantage of males (75% female and 25% male, Figure 4B, right panel). Given the high intrinsic variability of leukaemias, an increase in the number of stabilized cell lines and the isolation of new freshly isolated cells from males and females seem to be unpostponable.

Lymphoma

Lymphomas comprise a heterogeneous group of cancers with diverse aetiologies, treatment pathways and outcomes, varying both for the type of malignant cells and tumour location [53]. They most frequently originate from B-cells, and the two main groups of B-cell lymphomas, B-cell non-Hodgkin and Hodgkin, account, for ∼80% and 15% of all lymphomas, respectively [54]. As for many other cancers, the likelihood of an individual being diagnosed with lymphoma markedly increases with age, the median age at diagnosis being 67.2 years for all patients combined, as described by Smith et al. [55]. Hodgkin and Burkitt lymphomas dominate the paediatric age range (<15 years). By contrast, in those aged 60 years or more, diffuse large B-cell, marginal-zone and follicular lymphomas accounted for over 80% of diagnoses.

As concerns gender, males tend to be diagnosed with B-cell lymphomas at younger ages than females, the male/female sex rate ratio being highest in those diagnosed before the age of 15 years. Indeed, males are almost three times as likely to develop some B- and T-cell cancer subtypes [55]. However, when MEDLINE analyses was carried out considering preclinical studies performed on animals in this field (Supplementary Figure S1C), we found that
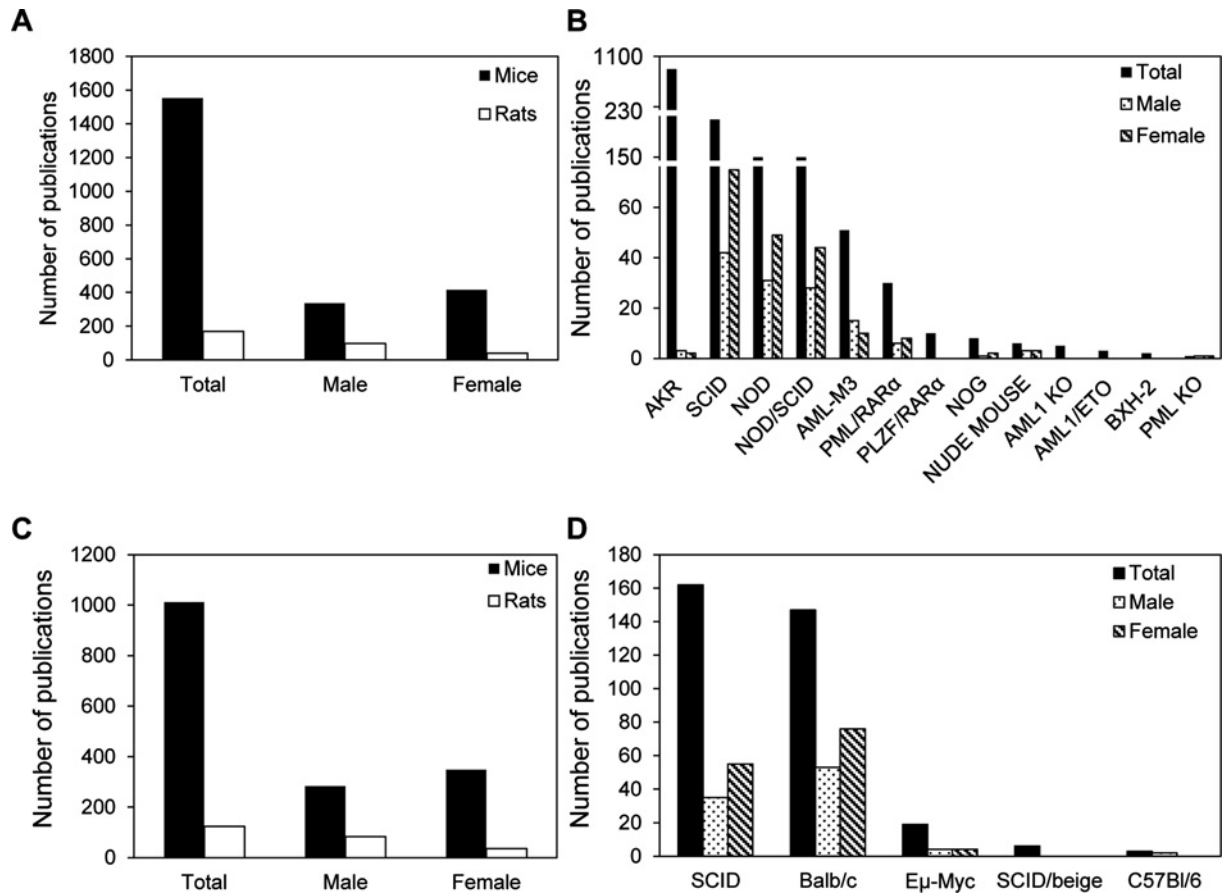
Figure 3. Leukaemia and lymphoma studies
(**A**) Number of publications shown in MEDLINE in the last 10 years where scientists report the use of mice or rats for leukaemia studies. (**B**) Here, we discuss only a selection of the currently available models considering the major signalling networks deregulated in leukaemia by mutation or other genomic alterations, which include: knockout models of AML1, PLZF and PML; xenograft models and third-generation immunodeficient hosts combining additional mutations on the NOD/SCID background. Newborn OD/SCID/Il2rg/('NOG') hosts support the highest level of engraftment to date for primary, unmanipulated human AML cells. (**C**) Number of publications shown in MEDLINE in the last 10 years where scientists report the use of mice or rats for lymphoma studies. The most used mice models for lymphoma studies are reported in (**D**).

differences reported above were ignored so that only male or female mice were taken into consideration. Figures 3(C) and 3(D) show the number of publications reported in MEDLINE in the last 10 years including mice or rats for *in vivo* lymphoma studies and the most used mice models for lymphoma studies. As far as the *in vitro* studies were concerned, following the same criterion of literature analysis through a specific search-string on PubMed, we selected the 10 most used cell lines in the literature among 70 cell lines commercially available (Figure 5A, left panel). These 10 cell lines represented 81% of the total cell lines used for preclinical studies on lymphoma. All of them were isolated between 1963 and 1983 from patients with a mean age of 26 y.o. The most commonly used is the Raji cell line analysed in 32% of the total number of the selected papers. Isolated in 1963 from an 11 y.o. male patient, Raji cells are lymphoblastoid derived from a Burkitt lymphoma and they are the first continuous human cell line of haematopoietic origin [56]. Also in the case of lymphoma, cell lines from males and females are not equally represented. In fact, 92% of the studies have considered cell lines from male patients only (Figure 5B, left panel). Restricting our analysis to the five most commonly used cell lines in lymphoma studies (top five represented 70% of published papers), this imbalance has become even more marked with this percentage reaching almost 100%. Of notice, DNA profiles for the aforementioned lines have not usually been counterchecked with the DNA profile of the donor tissue and compared with the DNA profile of other continuous cell lines provided by the data bank.
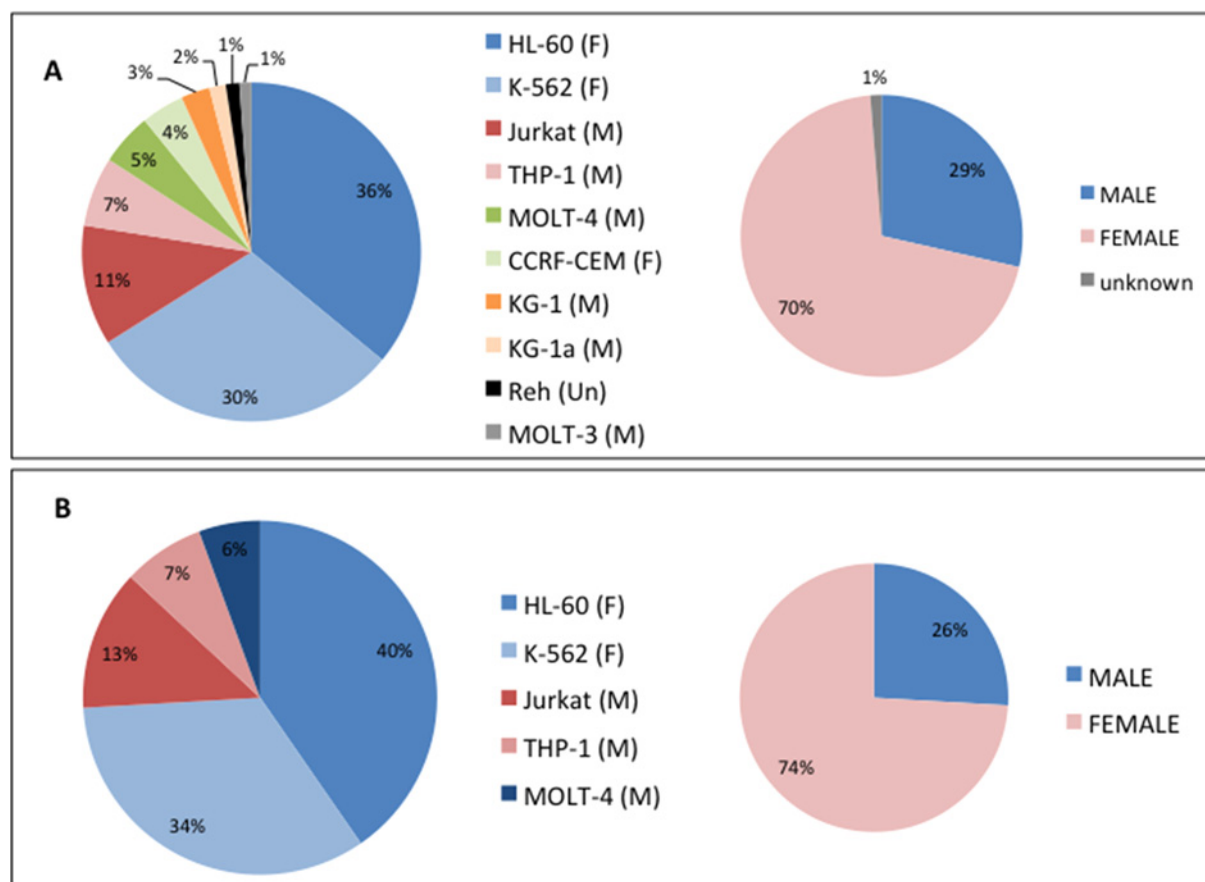
Figure 4. Most used cell lines for leukaemia studies

(**A**) Pie chart depicting the relative distribution of the scientific papers in which the 10 most used leukaemia cell lines have been used as an *in vitro* model (left panel). These 10 examined cell lines represent 92% of the cell lines used in the literature for *in vitro* studies on leukaemia. Pie chart depicting the sex distribution of the 10 most used cell lines in the literature (right panel). (**B**) Pie charts depicting the same analysis of panel A but restricted to the five most used cell lines. These five examined cell lines represent 82% of the cell lines used in the literature for *in vitro* studies on leukaemia.

All in all these bibliometric analyses in the field of cancer research underscore the fact that 'cell sex' is an under-considered issue. Only very recently, the work by Clocchiatti and colleagues raised this question in a leading journal possibly encouraging a sex-tailored research in the field of *in vitro* studies on cancer [17].

Neurological diseases

The interactive database GenderMedDB [38] showed an increased trend of neurological studies linked to gender disparity (Figure 6A). In particular, Alzheimer's, epilepsy, multiple sclerosis, Parkinson's and stroke are the most represented neurological diseases (Figure 6B). As for oncology, we explored sex differences in preclinical studies of two important, and very different, neurological diseases: stroke and Alzheimer's.

Stroke

Stroke, the second leading cause of death worldwide, is a cerebrovascular disease due to a focal neurologic deficit caused by interrupted or reduced blood cerebral circulation. According to the World Health Organization, 15 million people suffer stroke worldwide each year. Of these, 5 million die and another 5 million are permanently disabled. Europe averages ∼650000 stroke deaths each year and the overall rate of strokes remains high due to aging of the population. Approximately 610000 of these are first events and 185000 are recurrent stroke events. Control of diabetes mellitus and high cholesterol and smoking cessation programmes, particularly in combination with hypertension treatment, contributed to the decline in stroke mortality observed in the last years [57]. According to data from the 2013
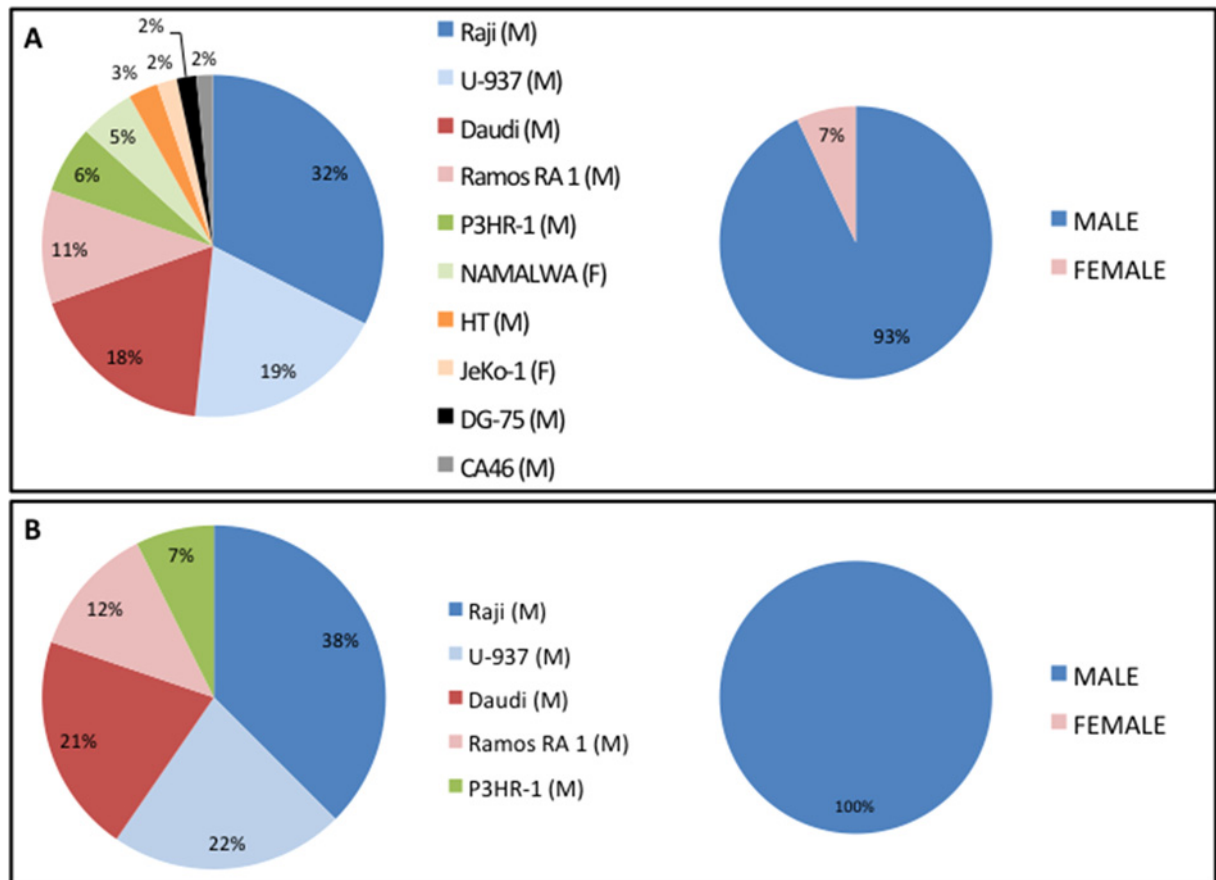
Figure 5. Most used cell lines for lymphoma studies

(**A**) Pie chart depicting the relative distribution of scientific papers in which the 10 most used lymphoma cell lines have been used as an *in vitro* model (left panel). These 10 examined cell lines represent 81% of the cell lines used in the literature for *in vitro* studies on lymphoma. Pie chart depicting the sex distribution of the 10 most used cell lines in the literature (right panel). (**B**) Pie charts depicting the same analysis of panel (**A**) but restricted to the five most used cell lines. These five examined cell lines represent 70% of the cell lines used in the literature for *in vitro* studies on lymphoma.

Behavioural Risk Factor Surveillance System, each year ∼55000 more women than men have a stroke. Epidemiologic studies have revealed a clear age-by-sex interaction leading to several mechanistic hypotheses. Stroke risk, symptom presentation and long-term recovery differ in males and females. In younger population, more strokes occur in men than women, but with advancing age (>60 year), in which stroke morbidity is higher, women have a greater risk with worse outcomes (reviewed in [58]). Premenopausal women appear less vulnerable to stroke than similarly aged men. However, in menopausal women, probably due to the lack of protective activity of oestrogen, prevalence and incidence of stroke are increased. Premenopausal women are most probably protected against stroke because of sex steroid hormone-dependent mechanisms. Oestrogen, testosterone and progesterone affect different functions of the cerebral circulation. Oestrogen promotes blood flow by decreasing vascular reactivity whereas testosterone has opposite effects. Both are involved in the development of atherosclerosis. Other factors, such as anatomic and genetic factors may also contribute to the observed differences [59].

Sex differences in response to experimental ischaemic stroke are also well established in animal models. Young females sustain smaller infarcts and better cerebral blood flow than age-matched males [60]. In a very recent work, it was reported that circadian rhythm disruption exacerbates these sex differences in stroke impairments because exposure to shifted light-dark cycles dramatically increased stroke-induced mortality in male but not female rats [61]. Ovarian hormones, especially oestrogen, may mediate the female advantage with regard to the lethal pathological effects of circadian rhythm disruption on stroke outcomes. This hypothesis is indirectly supported by studies demonstrating that infarct volume is increased in ovariectomized females relative to intact animals or oestrogen-replaced animals, and
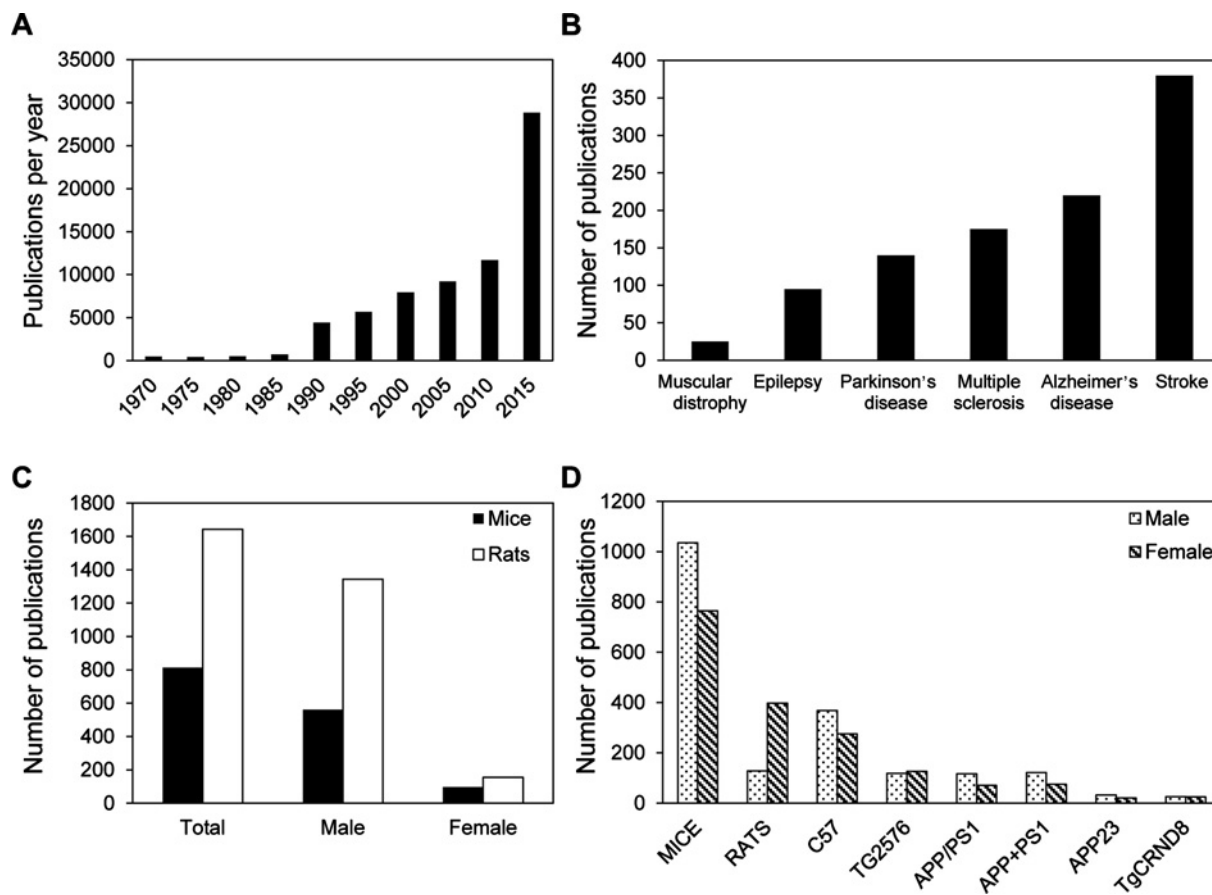
Figure 6. Neurological diseases studies

(**A**) Neurology-related publications per year (data from PubMed.gov). (**B**) Publication distribution within diseases (extrapolated data from GenderMedDB database). (**C**) Number of publications shown in MEDLINE where scientists report the use of mice or rats for stroke studies. The most used mice models in the last 10 years for AD preclinical studies are showed in (**D**) which include: C57Bl/6 wild-type mice; Tg2576 mice (the first presentation of cognitive deficits is seen at 5 months of age in spatial working memory); the APP23 mouse model (the cognitive deficits begin to first appear in both recognition memory and spatial working memory at 3 months of age, the deficits appear to be progressive with age); TgCRND8 model exhibits early cognitive impairment; the cognitive deficits in the APP/PS1 mouse model (cognitive deficits are first seen at 3 months of age in the RAWM spatial working memory task and are also reported by 6 months of age in the MWM); the APP/PS1 knockin (APP + PS1) mouse model uses endogenous promoters to drive the expression of humanized amyloid β sequence, and AD-like pathology and cognitive deficits develop in the absence of APP or PS1 overexpression.

that exogenous oestrogen treatment also promotes neuroprotection in response to stroke in males [62]. Other factors, in addition to oestrogen alone, could play a critical role in stroke neuroprotection observed in young females after circadian rhythm disruption. By the way, insulin growth factor-1 (IGF-1) plays a key role in modulating neuroprotective responses to stroke in young females. In fact, low levels of IGF-1 are associated with increased morbidity and mortality in ischaemic heart disease and stroke. In addition, exogenous IGF-1 reduces ischaemic injury, stimulates stroke-induced neurogenesis and promotes neuronal survival, neuronal myelination and angiogenesis [61].

Although women received comparable stroke care to men, as measured by specified 'Get With The Guidelines Metrics', other studies reported sex-specific disparities in stroke care, including thrombolytic therapy and early hospital admission [63]. A further possible cause that can contribute to the gender differences observed in stroke is the access to treatments. In fact, women are older at stroke onset and more likely to live alone, therefore evidencing longer prehospital delays after symptom onset compared with men [63].

In recent years, the use of animal models for stroke research has improved our understanding of the physiopathology of this disease. Rats and mice are the most commonly used stroke models, but the demand for larger models, such as rabbits and even non-human primates, is increasing in order to better understand the disease and its

treatment. However, the applicability of the results obtained with animals to the treatment of human diseases is limited. The use of older animals can provide information about stroke-induced damage and the recovery process, not well represented in younger animal models [64]. By using the MeSHs terms search (Supplementary Figure S1D), rats are more used than mice and male animals are underrepresented in most preclinical studies (Figure 6C). Importantly, the group of McCullough, that extensively studied gender disparity in stroke, strongly recommended performing stroke studies in animals of both sexes. These authors also underscore the relevance of this approach to point out gender-specific therapies [27,65]. In fact, much remains to be learned about differences in stroke between women and men and further research is needed to improve stroke risk profiles and gender-specific treatments.

Alzheimer's disease

Alzheimer's disease (AD) is manifested by a progressive loss of memory and cognition. It is the main cause of dementia and, in fact, ~60% of all cases of dementia are AD patients. The prevalence of AD is higher in women than in men. The prevalence of Alzheimer's disease in Europe was estimated at 5.05%. The prevalence in men was 3.31% and in women 7.13% and this gender disparity increased with age. This sex difference may be due to the higher longevity women generally experience. However, actually, the incidence also differs between men and women: it has been found to be 7.02 per 1000 person-years (95% CI, 6.06–8.05) in men and 13.25 per 1000 person-years (95% CI, 12.05–14.51) in women; again these rates increased with age [66]. In fact, increasing evidence suggests that longevity alone is not a sufficient explanation and other factors may be involved. Women have a broader spectrum of dementia-related behavioural symptoms with a predominance of depression, whereas aggressiveness is more frequent in men than in women. Biological explanations for gender-specific differences in the phenotype of AD include different brain morphology and function, with higher susceptibility for pathological lesions in women and greater cognitive reserve in men [67]. A further hypothesis for the higher incidence of dementia in women is that they suffer higher rates of obesity, diabetes and other conditions, which increase the likelihood of developing AD [68]. However, the normal age-related depletion of sex steroid hormones also represents an important AD risk factor. Steroid hormone decrease is associated with vulnerability to disease in all the hormone-responsive tissues, including the brain. Few studies have addressed the effects of aging on brain hormone levels [69,70]. The literature suggests that both loss of oestrogen and progesterone at menopause in women and the gradual decrease in testosterone in aging men could be AD risk factors. As such, therapeutic strategies that counteract age-related depletion of sex steroid hormones may offer significant protection from the development and perhaps treatment of AD [71]. Further points of interest also derive from a very recent study pointed to children's air pollution exposures as associated with systemic and brain inflammation and the early hallmarks of AD [72]. These authors indicate the Apolipoprotein E (APOE) 4 allele as the most prevalent genetic risk for AD, with higher risk for females, suggesting that sex, BMI, APOE and metabolic variables in healthy children with high exposure to ozone and fine particulate matter (PM2.5) influence cognition, with glucose probably being a key player.

Preclinical animal models, especially in mice, have been extremely useful to test mechanistic hypotheses about AD pathophysiology and to predict outcomes from pharmacological interventions. However, no animal model recapitulates the entirety of AD in humans, thus making it important to understand both the utility and limitations of particular animal models. For instance, the progression of cognitive impairment seen in human diseases was not detectable in AD mice models. Each transgenic (Tg) mouse model of AD provides different insights into aspects of AD pathogenesis and the cognitive deficits associated with the disease. In particular, animal models used in preclinical studies for AD, including Tg models in mice, and non-Tg models also in rats, dogs and monkeys can be distinguished in: (i) Tg models of AD, consisting of single or multi-Tg animals overexpressing the amyloid precursor protein (APP), presenilin (PS) and/or Tau mutations; (ii) non-Tg models obtained by toxin injection into the brain, including direct injection of Aβ oligomer or tau, and models of aging. The most commonly used mice models of AD are characterized by APP mutations [73]. Moreover, it could be useful to note that C57Bl/6 represents the most diffuse wild-type background of the mouse Tg models [74]. As observed, male mice are often used more often than female mice (Figure 6D). 5XFAD mice, a well-characterized double Tg mouse model of AD, exhibit an early onset of robust AD pathology and memory deficits, thus representing a good animal model to investigate AD pathology. These mice have five distinct human mutations in two genes, the APP and presenilin1 (PS1) engineered into two transgenes driven by a neuron-specific promoter (Thy1), and thus develop severe amyloid deposition by 4 months of age. In particular, BACE1·5XFAD haploinsufficiency [β-Site APP-cleaving enzyme 1 (BACE1) ($+/-$); i.e. 50% reduction] represents a therapeutic relevant model to evaluate the efficacy of partial β-secretase inhibition. BACE1 initiates the generation of amyloid-β (Aβ), thus representing a prime therapeutic target for AD. However, it is unclear whether the

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extent of Aβ reductions in APP Tg mice with BACE1 (+ /-) gene ablation may vary with sex or disease progression [75].

Synapses appear to be the initial target of AD pathogenesis; as loss of synapses at a fine structural level as well as reduction in synaptic markers have been documented at early and late stages of AD [76] and shown to correlate with the extent of cognitive deficits [77]. Progressive decline of synaptic proteins occurs also in the 5XFAD mouse model and appears to be correlated with the progression of memory deficits [78]. For instance, by using this model it has been documented that along with the improvement of cognitive abilities, neuronal matrix metalloproteinase-9 (MMP-9) overexpression was accompanied by increased levels of the pre-synaptic marker synaptophysin in the brain of 5XFAD female animals. MMP-9 has been shown to participate in receptor-mediated sAPPα release [79] and to be able to degrade Aβ fibrils *in vitro* and Aβ plaques *in situ*. Consistently, it was demonstrated that overexpression of MMP-9 restored the levels of mature brain-derived neurotrophic factor (BDNF) in the brains of 5XFAD females. Such an increase could contribute to the preservation of cognitive functions observed in 5XFAD/TgMMP-9 animals.

As far as the *in vitro* model systems are concerned, although there are many *in vitro* studies on the topic of neuropathology, particularly on AD, a careful analysis of the cell lines used in the field of neurodegenerative diseases clearly indicated that the authors do not specify if cell lines or primary cultures were derived from males or females. A number of mechanistic studies on AD are achieved by using the human neuroblastoma cell line SH-SY5Y, genetically female with two X and no Y chromosome (Table 2). This cell model has two important features that make it eligible for this purpose: (i) following retinoic acid treatment, it acquires a neuronal phenotype evidenced by axonal growth and expression of neuronal markers and (ii) it is easily transfectable, therefore suitable for exploring the effect of different genes, i.e. the APP gene, involved in the pathogenesis of AD. However, these are highly proliferating tumour cells and, as usual, they are cultured cells that have experienced a number of subseedings without any check of their karyotype. The same occurs for another cell line, the PC12 cells, derived from a male rat pheochromocytoma, widely used for studies on neuroprotection. For example, PC12 cells were used as a preclinical model to investigate some forms of Parkinson's disease with finds that support interference after vesicle docking and prior to vesicle release [80]. These studies suggest that, in women, higher physiological striatal dopamine levels may delay the development of symptomatic PD and that this is possibly due to the activity of oestrogens. This could explain the epidemiological observations of lower incidence and higher age at onset in women. However, also in this case, the model system, although appropriate, deals with tumour cells from rat adrenal medulla that, having an embryonic origin from the neural crest, only partially mimic neuronal pathophysiology. Primary cultures of neurons should be considered in order to correctly investigate neurodegenerative diseases, especially in sex-oriented research.

Cardiomyopathy and heart failure

Cardiology is of critical importance and represents a milestone in the field of GM. It has in fact been investigated for many years [1,81,82]. Major differences between men and women exist in epidemiology, manifestation, pathophysiology, treatment and outcome of CVD [83,84]. Women have less cardiovascular disorders than men in the premenopausal period with risks increasing in the postmenopausal period reaching and even exceeding that of men [85]. In addition, women have more frequent diastolic heart failure (HF), associated with the major risk factors of diabetes and hypertension whereas men have more frequent systolic HF because of coronary artery disease [79]. Under stress, male hearts develop pathological hypertrophy with dilation and poor systolic function more easily than female hearts [86]. As far as acute myocardial infarction (AMI) is concerned, females, at least in some studies, have been reported to experience a longer delay to reach hospital and less frequently receive invasive therapy, e.g. stents, and cardiac bypass surgery, than males [87]. For both sexes, age represents a demographic predictor of short- and long-term mortality in AMI [88]. An analysis from the American National Registry of Myocardial Infarction underlined an interaction between sex and age with regard to 30-day mortality. In particular, this parameter was significantly higher in females. Interestingly, there was a progressive decrease in the male/female difference with increasing age until the age of 75 years [89,90]. A very recent study based on retrospective collection of data compared females and males over the age of 75 years with regard to the diagnosis of AMI and survival follow-up data for more than 7 years [91]. In contrast with that observed with regard to 30-day mortality, after 7 years mortality was as high as 80% for both sexes, but males had a significantly higher age-adjusted 7-year mortality than females [91]. These are just some of the sex differences observed in cardiovascular disease onset and features.

As concerns preclinical studies with animals, sexual dimorphism is a well-established phenomenon [92]. However, its degree varies dramatically among species. In fact, for example, the aim of animal modelling of HF is to simplify an extremely complex syndrome into more manageable research questions. A key decision is the choice of the animal system, which is often a trade-off between convenience/cost and physiological applicability. Usually, animal

studies use single sex homogeneous groups maintained on standard diets in carefully controlled environments and this makes these models not suitable to detect sex differences. The relatively complete annotation and simple manipulation of the mouse genome have allowed significant mechanistic insights into human disease. Although mice are relatively inexpensive and convenient, there are significant differences between mouse and human hearts that lower the relevance of preclinical analyses. Mouse hearts are obviously very small and beat very quickly [400–600 beats per minute (bpm)] compared with humans (60–90 bpm), leading to important differences in calcium handling and ion currents between mice and human hearts. There are also important differences in the predominant myosin isoforms expressed in adult human and mouse hearts [93]. Human data indicate that sex differences are particularly noticeable at lower heart rates, so unsurprisingly, data on sex differences in mice are difficult to interpret overall in the field of electrophysiology. The dog, with a heart size and cardiac action potential (AP) characteristics similar to humans, has been extensively used as a model for HF and sudden cardiac death. However, in contrast with humans, sex differences have not been observed in the Corrected QT Interval (QTc) or other ECG parameters in this animal model [94]. This issue is of importance as we have known for many years that there are marked differences in QT intervals of men and women. In addition, only recently we also appreciated the profound implications of sex-based electrophysiological differences in QT interval on the shown sex differences with respect to arrhythmia risk, drug sensitivity and treatment modalities. Little is known about the fundamental mechanisms responsible for sex differences in the electrical substrate of the human heart, in large part due to the lack of tissue availability. Although animal models represent an important research tool, species differences in the sexual dimorphism of the QT interval, the ionic currents underlying the cardiac repolarization and effects of sex steroids advice against the possibility to extrapolate sex-related results from animals to humans. In fact, studies performed on different animal models often yield conflicting data. Each model has its strength, such as ease of genetic manipulation in mice or proper size in dogs. The New Zealand white rabbit is a frequently used animal strain. It exhibits similar sex differences in LQT2-related arrhythmias as in humans, for both adult and prepubertal rabbits, and approximately the same combination of ionic currents underlie rabbit and human AP [95].

As concerns HF, the most commonly used mice models use the response to a surgical intervention, such as banding an aorta or clipping a coronary artery, to model the multisystem effects of HF [96]. These surgical models of HF have the advantage of very closely replicating specific disease situations of myocardial infarction (coronary artery ligation) and HF owing to hypertension or aortic valve stenosis (aortic banding). However, all surgical models are relatively expensive and technically demanding with high rates of intraoperative mortality, which reduces reproducibility. Interventions such as long-term pacing of the heart at high rates are usually used in larger animals, for example dogs and rabbits, but this is not technically feasible in mice. Administration of a single toxin or drug is a theoretically attractive method of modelling HF. For example, the antineoplastic drug doxorubicin [97] is known to be cardiotoxic in humans and has been used in mice to induce HF syndromes in which, as in humans, a sex disparity has been detected [85]. An additional useful approach offered by mice models could be represented by the changes in expression of some genes involved in HF by targeted mutagenesis/transgenesis. Unfortunately, some lines of evidence demonstrate that diverse gene expression patterns are associated with HF due to differing causes [98]. Thus, the deletion of a single gene has a high chance of leading to artifactual phenotypes not representative for human disease [99].

Further, large animal models that approximate human physiology, function and anatomy, recapitulating the clinical HF phenotype and translating basic science to clinical applications have successfully been used. In particular, due to anatomical dimensions, for cardiac mechanical devices and surgical procedures, swine are recognized as the most suitable animal model also for clinician training in optimizing new technical procedures [100]. However, the high economic costs and the difficulties of stabulating make this animal model unsuitable, also from the point of view of statistical significance.

Analysing literature of the last 10 years in the MEDLINE database incorporating MeSH® of HF, by using the list of strings shown in Supplementary Figure S2(A), we found that most of the studies dealing with HF used rats or mice. In particular, the use of male mice and male rats is higher than female small size animals (Supplementary Figure S2B). Currently, the limiting factor is the size of the mouse heart even if new technologies in molecular imaging of mouse hearts are developing in parallel with human technology. Validation of the effect of drug treatment in mice models against human CVD remains therefore of primary importance.

As far as the *in vitro* model systems are concerned, as mentioned in the neuropathology section, even in the case of CVD, it was not possible to make an accurate analysis of the cell lines most used in experimental research. A number of studies in the CVD field used the H9c2 rat cardiomyoblast cell line, which has the advantage of being an animal-free alternative. A careful evaluation by Watkins et al. [101] demonstrated that H9c2 cells could accurately mimic the hypertrophic responses of primary cardiac myocytes. This finding validates the importance of H9c2 cells

as a model for *in vitro* studies of cardiac hypertrophy and supports current work with human cardiomyocyte cell lines for prospective molecular studies in heart development and disease [101]. In spite of this, there is no information on grounds of sex of this important cell line (Table 2), which originates from myocardium of *Rattus norvegicus* [102]. However, *in vitro* differentiated human cardiomyocytes still represent an essential tool not only for general cardiovascular research, but also to address key unmet needs in drug development and preclinical research. New testing systems, such as induced pluripotent stem cell (iPSC)-derived cardiomyocytes, enable the assessment of the safety pharmacology core battery at the preclinical stage [103,104]. In fact, a major constraint for the development of adequate therapies has been the lack of suitable cell-based assays with physiological relevance. Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) and higher throughput platforms have emerged as potential tools to advance cardiac drug safety screening, in particular to examine the effects of compounds on cardiac electrical activity. Although this model system has been validated for many applications (among these calcium transient analysis, voltage sensitive dyes, cell metabolism analysis, hypertrophy disease modelling, cell contraction force and 3D organotypic cell culture), currently only hiPSC-CM originated from a female are available (Table 2). Thus, also in this area of research, the *in vitro* models used so far appear inadequate for sex-oriented studies.

Conclusions
The experimental matter
As recently suggested by some papers analysing literature data, very few works take into account both males and females in experimental settings or discuss the problem of sex differences. Preclinical studies, either carried out *in vitro* or *ex vivo* or in animal models, barely consider both sexes. More often only one sex is taken into account. In addition, frequently, the analysed experimental models do not reflect the incidence in humans so that a disease, which is predominant in females, has been analysed in preclinical studies carried out in male mice or *vice versa*. A paradigmatic example is that of pharmacological studies in the field of neurodegenerative diseases, more frequent in females but studied mainly in males.

The editorial needs
The fundamental role of editors in disseminating research output is well evident. The rules and policies of journals can directly affect research. For example, ethical review procedures are now a universal requirement for human and animal research at least in part because of journal editorial requirements. In the same vein, a specific work [105] proposed a series of recommendations aimed at the inclusion of information regarding sex in the context of the work. For example, papers should specify the sex of animals or cells, tissues and other material derived from these, or the sex/gender of human participants. Authors should report how sex/gender was taken into account in the design of the study, whether they ensured adequate representation of males and females, or justify the reasons for any exclusion of males or females. Altogether the required information could provide useful clues in the development of the biology of sex differences and GM and the improvement of our knowledge of disease onset and progression. Nowadays, information on the sexual origin of cells or animals is too often disregarded so that the great majority of works still lack this important information either in *in vitro* or in animal studies. As a consequence, comparisons between the two sexes are very rare and almost barely investigated and discussed. We hope that this brief discussion on the state of the art of the models used in different fields of experimental research contributes to a reappraisal of the 'sex issue' in preclinical studies.

Abbreviations
AD, Alzheimer's disease; ALL, acute lymphocytic leukaemia; AML, acute myelogenous leukaemia; AP, action potential; APOE, Apolipoprotein E; APP, amyloid precursor protein; BDNF, brain-derived neurotrophic factor; CLL, chronic lymphocytic leukaemia; CML, chronic myelogenous leukaemia; CVD, cardiovascular disease; EBV, Epstein-Barr virus; GM, gender medicine; HF, heart failure; HPV, Human Papilloma virus; iPSC , induced pluripotent stem cell; IGF-1, insulin growth factor-1; MeSH®, Medical Subject Headings; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; PS, presenilin; Tg, transgenic.

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