SDIRSACR

Official Journal of the Serbian Association for Cancer Research
Grahovac J. Oncol Insights 2023 No. 1
613.88:616-006.6
616-006.6-085.85

Oncology Insights

REVIEW ARTICLE *Open access*

The importance of sex as a biological variable in cancer research

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Abstract
Sex is an important biological variable that has an impact on all aspects of human health and disease. Yet, it is greatly unappreciated in both basic and translational cancer research, and most concerningly in cancer clinical trials. In this review we summarize how patients' biological sex influences cancer risk, the biology of cancer and its response to anticancer therapy. We present data from the past decade on the genetic, genome-wide, metabolic and immune differences between sexes and how they relate to cancer development and progression. Ultimately, we highlight the importance of considering sex as a variable in all aspects of cancer research and recommend guidelines for implementation.
Keywords: sex, bilogical variable, genetics, metabolism, immunity

Introduction

There is growing evidence that non-reproductive cancers are initiated earlier, associated with higher incidence and greater mortality in males than in females (1). Hormones play a role in observed sex differences in cancer incidence and outcome, however in recent years it has become apparent that genetic and epigenetic foundations are equally important (2, 3). Factors that affect metabolism, immune response and response to therapy also differ by sex. Females generally have better response to treatment, yet it is associated with higher toxicity (4).Despite the evidence from both basic and translational research that implies that sex bias in cancer exists, it is most commonly overlooked. In this review we will highlight the significance of including sex as a variable in all stages of cancer research from early cell and animal testing to clinical trials; and underline the importance of sex segregated analysis that will lead to novel discoveries and improved personalized treatment for cancer patients.

Sex and gender defined

Many researchers are still unfamiliar with the distinction between sex and gender. In humans, sex refers to the biological and physiological attributes that distinguish male, female and/or intersex (5). Sex chromosomes, hormones and reproductive organs serve as biological determinants of sex. Genetic sex refers to XX and XY chromosomes that are present in every cell in our bodies, therefore all cells have sex (6). The impact of sex on human health is dynamic and changes throughout life. In biomedical research, the factor of sex deserves an integrative approach (7) as important differences between males and females exist in body composition (percent of fat and muscle), hormonal status, metabolism, immunity and pharmacokinetics and pharmacodynamics of drugs. Gender refers to sociocultural attitudes, behaviors and identities. Gender attitudes and behaviors can change with time and place, vary from society to society, and can intersect with sex, age, socioeconomic status, sexual orientation and ethnicity (5). Gender differences in cancer can only be studied in humans and cannot be modeled in preclinical models. Important gender differences in cancer are, for example, exposure to risk factors, care seeking and therapeutic choices. In this review terms male and female will refer to the biological sex.

In most cancer types, males have higher incidence and higher mortality rate compared to females (2) (Figures 1 and 2). Despite having higher incidence rates in some types of cancer like breast, thyroid and gallbladder for example, females have better survival than males. This implies that fundamental biology of sex differences affects cancers of all types (8).

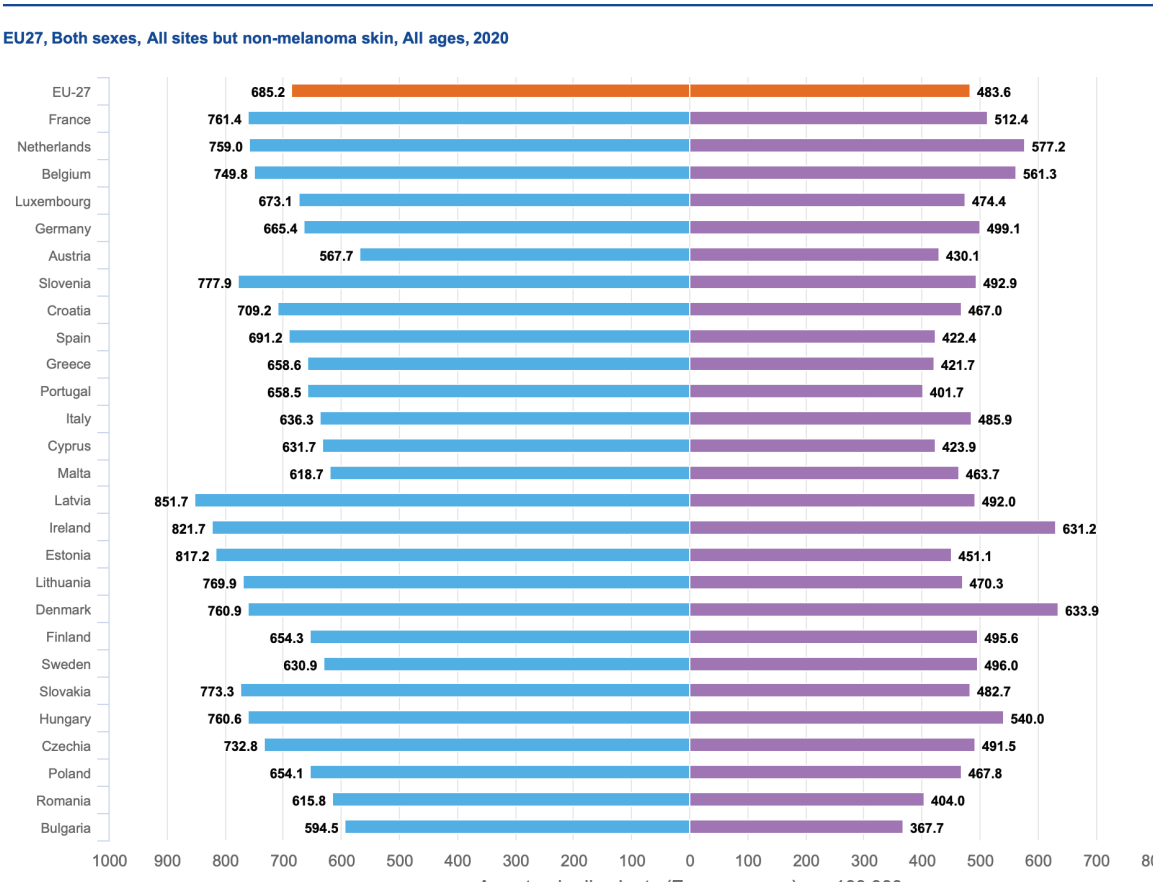

Figure 1. Estimates for cancer incidence per country from the European Cancer Information System (ECIS) From https://ecis.jrc.ec.europa.eu, accessed on 15/09/2023 © European Union, 2023.

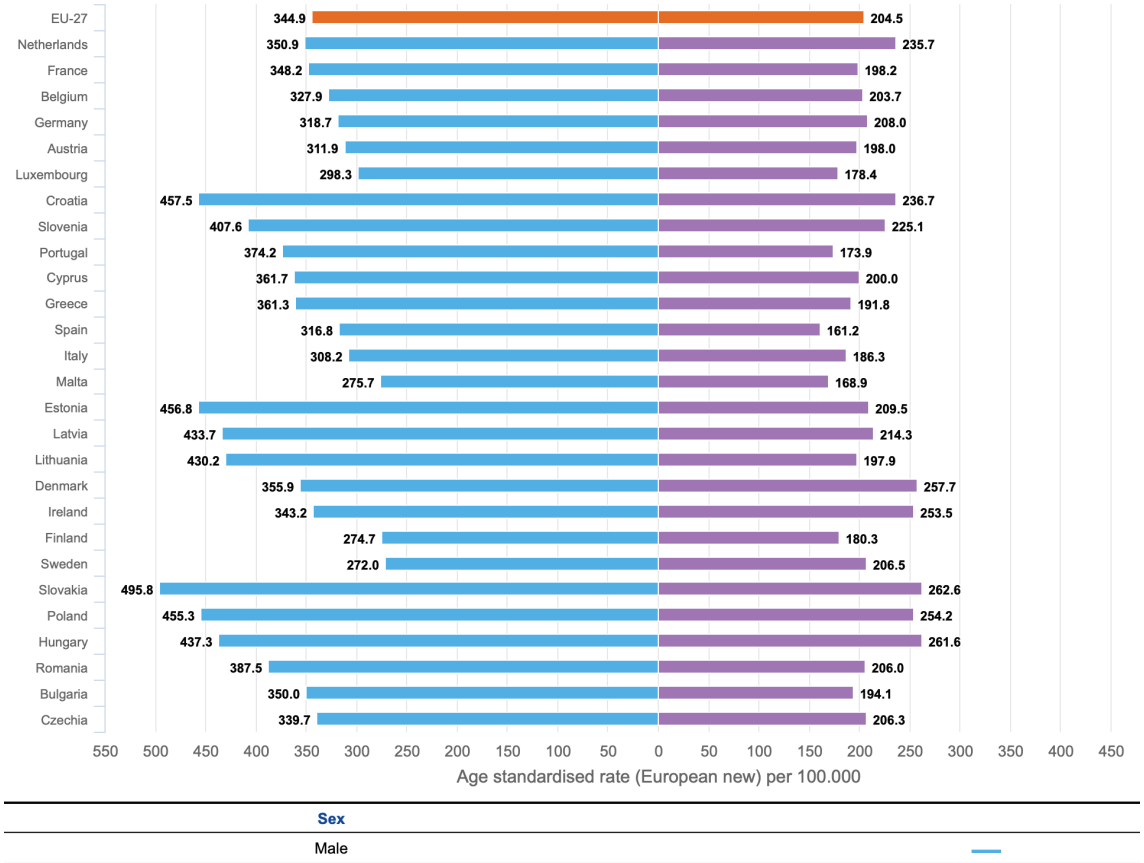

Figure 2. Estimates for cancer mortality per country from the European Cancer Information System (ECIS) From https://ecis.jrc.ec.europa.eu, accessed on 15/09/2023 © European Union, 2023.

Genetics of sex disparity in cancer

The X chromosome

Molecular mechanisms driving sex differences in disease are poorly understood and most approaches in precision medicine assign therapy without considering sex as a variable. Genetic and genome-wide sex differences influence both cancer biology and outcome. As X chromosome inactivation is incomplete, some genes can be expressed from both alleles in females, resulting in important sex bias based on sex chromosomes (2). A portion of the reduced cancer incidence in females as compared to males across a variety of tumor types has been attributed in part to the male-biased mutations in genes that escape X-inactivation. In more than 20 cancer types higher mutation rate in males was identified for 6 tumor suppressor genes termed EXITS – escape from the X-inactivation tumor suppressors (9). Two X-linked genes that can escape X-inactivation have a role in immune response – FOXP3 (10) and CD40L(11) – are associated with increased susceptibility to autoimmune disease in females, but may present an advantage in anti-tumor response. Numerous genes that engage in p53 networks are also located on the X chromosome. This is of importance as four key processes that link to cancer sex disparity: immune response, regulation of apoptosis and cell cycle, metabolism and DNA repair are linked to the tumor suppressor p53. In males, there are also higher frequencies in p53 mutations (12). Chromatin accessibility is as well strongly dependent on patient sex, especially on the X chromosome (13, 14). Further support for the effect of biallelic X chromosome gene expression on cancer development comes from a study showing that women with Turner's syndrome (X chromosome monosomy) have increased risk for solid tumor development compared to XX women; and men with Klinefelter syndrome (with two or more X chromosomes) have lower risk for solid tumor development compared to XY men (15).

Sex bias in gene expression and mutational burden

Comprehensive analysis of molecular-level differences between male and female cancer patients in 13 cancer types revealed both sex-biased gene expression patterns for more than 60 individual genes and sex-biased molecular signatures comprising of more than a thousand of genes per signature (16). Importantly, more than 50 percent of clinically actionable genes showed sex-biased expression. For example, a major therapeutic drug target in lung adenocarcinoma, EGFR, showed female-biased mRNA expression (16), that may have contributed to the better response to the EGFR inhibitor erlotinib in female patients (17).

Large differences between the two sexes in mutation density and in the frequency of mutation of specific genes in cancer were also reported; these were suggested to be associated with sex biases in DNA mismatch repair genes and microsatellite instability (18). The first study that investigated genomic differences underlying sex bias was on metastatic melanoma, where authors found that male patients had significantly more missense mutations than female patients (19). Interestingly, somatic mutations have accumulated earlier in the life span in males than in females, implying that the differences in ageing rates account for at least a part of the observed bias (20). The differences in mutational sex bias, however, were not common across tumor types. Some tumor types showed sex-biased differences in SNV mutation profiles, others in CNA mutational profiles, and some in both. Sex-biases existed in both coding and non-coding cancer drivers (21). The mechanisms behind these differences remain to be elucidated.

It is often hard to reach statistical power in sex-stratified analysis of genetic variants (22), nevertheless they can reveal important disparities in cancer. A single nucleotide polymorphism in a negative regulator of p53, MDM2, has been shown to increase cancer risk in several cancer types in females but not in males (23). Many genes related to drug metabolism have genetic variants that impact males and females to a different extent. These can be of influence in the clearance of chemotherapy and associated toxicity.

Sex-biased methylation patterns have been observed in many human tissues, influenced by the presence of the sex chromosomes and sex hormones. Genome wide analysis in 13 cancer types also reported sex-dependent methylation patterns, where most of the genes that had sex-biased methylation had sex-biased expression (16). It is important to note though, that very few methylation studies have included X and Y chromosomes in the analysis.

Taken together, sex biases in activity, repair and folding of the human genome are associated with differences in cancer incidence and outcome between men and women (24). Including the equal number of male and female patients in studies, and segregating the analysis by sex is of utmost importance in the era of high-throughput sequencing and genome wide association studies. Although higher incidences in males dictate the bias, one can design to prospectively collect or retrospectively analyze equal number of patients per sex.

Sex-related differences in cancer metabolism

Many metabolic processes differ between healthy females and males. Both gonadal hormones and X-linked genes contribute to glucose and lipid metabolism and obesity (25). Consequently, associated disease risks differ between sexes (26). In the 13 non-reproductive cancers from the TCGA database significant sex differences in glycolysis, bile acid and fatty acid metabolism were observed (16). High blood glucose levels are associated with higher prevalence and mortality in cancer, as high glucose promotes cell proliferation, invasion and migration, induces the apoptotic resistance and can enhance the drug resistance in tumor cells (27, 28). Fasting hepatic glucose uptake is generally higher in males than

in females (29), and increased blood glucose concentration is associated with higher cancer risk in liver and colorectal cancer in males but not in females (30, 31). Using transcriptome analysis, male-specific decreased survival related to glycolytic gene overexpression was found in patients with glioma (32). Further evidence that glucose metabolism has sex-biased impact in cancer outcomes comes from the meta-analysis of 8 cohort studies on colorectal cancer (CRC). This study revealed that while metformin (glucose levels reducing drug) decreased overall mortality of CRC patients with Type 2 diabetes mellitus, females with T2DM using metformin had a lower CRC-specific mortality than males (33). There are 435 registered clinical studies on metformin for repurposing in cancer treatment, with 82 currently recruiting (clinicaltrilas.gov accessed on 15.09.2023) and one can only assume that analysing treatment outcome segregated by sex would lead to novel insights.

Both diabetes and obesity are associated with increased cancer risk (34). Visceral adiposity is higher in men, while subcutaneous fat accumulates more in females (35). Visceral fat is a source of pro-oncogenic adipokines that especially contribute to the development of hepatocellular cancer (36). While it is recognized that lipid metabolism and obesity are associated with inflammation that disproportionately increases cancer risk in males (37), sex-segregated studies that will elucidate the exact mechanisms behind this disparity are still lacking.

Sex disparity in cancer immune response

Sex is a biological variable that strongly affects the immune system, in both innate and adaptive response. Sex chromosomes and sex hormones (as well as nutrition and microbiota) regulate differential response between females and males (38). In adults, differences in lymphocyte subsets including B cells (higher in females), $CD4^+$ T cells (higher in females), $CD8^+$ T cells (higher in males) and CD4/CD8 ratios (higher in females) are well documented (38). Furthermore, activity of CD4 subsets also differs between sexes. The difference in mounting an inflammatory immune response between the sexes translates into difference in immune defense against cancer (37). It was recently shown that male $CD8^+$ T cells exhibited impaired effector and stem cell-like properties compared with female $CD8^+$ T cells, where androgen receptor inhibited the tumor-infiltrating $CD8^+$ T cell activity by regulating transcriptional programs epigenetically (39).

Around fifty genes that are involved in innate and adaptive immunity are X-linked (40). More robust and heterogeneous immune response in females gives an advantage in anti-tumor response during cancer development. On the contrary, sex dependent mutational burden and sex dependent inherent immune surveillance differences give males an advantage in response to immune checkpoint blockade therapy. Immune response in tumors in males and females should be studied separately, from ***in vitro*** assays using both female- and male-derived model systems to informed patient selection strategies that will better hone immunotherapy approaches.

Sex differences in response to cancer treatment

Drug development has historically followed one size fits all model. However, there are significant differences in therapeutic response and toxicity in male and female cancer patients. Given the body composition, women often receive greater relative dose, that may translate into greater toxicity, but also better response. In the phase II and III clinical trials for chemotherapy, immunotherapy and targeted therapy, spanning four decades and including more than 23,000 cancer patients (excluding sex-specific cancers), women had 34% increased risk of severe adverse effects from therapy (4). Particularly large differences were observed for patients receiving immunotherapy. It is important to note that in these combined 202 analyzed trials women were presented with 37.9% of patients. Indeed, systemic review of randomized immunotherapy clinical trials in the last 10 years showed that women were strongly under-represented (41). Patient response to the immune checkpoint blockade therapy (with inhibitors of PD1, PDL1 and CTLA4) showed divergent patterns for sex bias (42). Women had better response rates in non-small cell lung carcinoma compared to males, but males had better response in colorectal cancer and in six out of seven clinical trials in melanoma (42). Elucidating the opposing sex disparities in response to immune checkpoint blockade demands adjustment for further confounding factors that differ between these cancer types, such are smoking, tumor purity, and age at diagnosis. Inclusion of sufficient number of females in these trials is also fundamental, as mentioned above.

Very few studies investigated response to radiotherapy treatment by sex. Radiotherapy offered advantage in females at the expense of toxicity in oesophageal squamous cell cancer, while underlying biological mechanism was not investigated (43). In this study cardiac toxicity occurred at significantly lower doses in females than in males. Toxicity of chemotherapy is also higher in females. This is a consequence of sex- related differences in pharmacogenomics, pharmacokinetics and pharmacodynamics of drugs in males and females (44). Women have consistently worse safety profile with slower processing of most drugs, higher accumulation of lipophilic drugs, decreased gastric motility, stomach acidity and kidney excretion, which result in slower excretion and elimination of therapeutics. In spite of these known differences, most treatment strategies do not account for sex. However, despite the toxicity, females survive longer than males in response to most of the chemotherapeutics: DNA alkylating agents, antimetabolites, antimitotics and anticancer antibiotics (37).

Existing policies and recommendations

In 1993 National Institutes of Health (NIH) in the United States mandated the enrollment of women in human clinical trials and twenty years later demanded the same in preclinical investigation – to be performed in both male and female animals (45), as several surveys showed that in many biological disciplines researchers used disproportionately higher number of male animals. European commission adopted similar policies in 2014. Mandated policies raised concerns that including both sexes in research will waste resources and slow down research (46). Others have pointed out that the costs of not taking sex into account are even higher as they result in failed clinical trials, misdiagnosis and inappropriate therapies for women and omission of fundamental biological principles (7). Unfortunately, even today not all researchers are fully aware nor they adhere to these recommendations in cancer research. Zucker and Beary analyzed almost 2000 animal studies and found a male bias in 8 out of 10 biological disciplines (47). It was often assumed that results from male animals applied to females, and studies where both sexes were included frequently failed to analyze results by sex. Lack of interest in sex differences is harmful but also presents a missed opportunity for innovation (48). How taking sex as a biological variable has led to novel discoveries can be seen in the recent years. For example, untargeted metabolomic analysis of colon tumors segregated by sex revealed sex differences in energy production and amino acid metabolism and helped define a novel subphenotype in women with right-sided colon cancer with implications for stratifying patient outcome (49). In a murine model of colon cancer with inducible KRASG12D mutation and conditional null alleles of APC and p53 tumor suppressors, male animals had higher metastatic rate and worse outcome (50). This finding led to a discovery that a Y-chromosome coded histone demethylase down-stream of mutated KRAS decreases tight junction integrity in cancer cells making them more prone to migration. By analyzing data segregated by sex, our group has reported that in melanoma tumor suppressor nischarin had positive prognostic value only in female patients, while in males it was associated with tumor B cell infiltration and negative patient outcome (51). This will have implications on the potential for repurposing of nischarin agonists for treatment of melanoma.

Many a time failure to translate research findings from basic to translational research to therapeutic benefit were blamed on issues of subjective bias, inappropriate experimental design and statistical analysis as causal factors. But more recently, it was acknowledged that studying predominantly one sex also contributes to failure to translate. Some journals have recognized this importance and have changed the guidelines to demand that sex is no longer ignored as a biological variable (52). This editorial noted that while the reporting of sex is encouraging, it is not enough to state the sex of the cells, animals and human specimens analyzed, but to improve practices further, they encouraged researchers to analytically study both sexes. European Union policy review on how inclusive analysis can improve research and innovation (5) suggests that all studies in humans and animals should consider whether sex is a covariate, confounder, or explanatory variable and report sex even at the level of cells and tissues used in research. Namely, female and male cells can exhibit sex differences in transcriptional profile in culture, as well as differences in growth rate, metabolism and response to stimuli (53).

Conclusions

Parameter of sex is largely omitted in both basic and translational cancer research spanning from cell line testing, validation in *in vivo* models in mice and zebrafish, as well as in patient biopsies. Most researchers do not consider sex specificity in study design and interpretation and molecular mechanisms underlying sex bias in cancer remain largely unknown. Females are largely underrepresented in both clinical trials and animal studies, and results are often generalized to both sexes based on research performed on males. It is essential to consider sex in every stage of preclinical and clinical research to improve prevention, diagnosis and treatment for all cancer patients. In both research and clinical practice sex should be used as a stratifying factor and sex-specific analyses should be performed. These considerations could lead to novel findings and development of treatments that increase efficacy while limiting toxicity.

In the last decade, a new concept has emerged that tumor types with the same histopathological phenotype may have distinct molecular etiologies in men and women (24). Identifying differences between the sexes will be of utmost importance in improving cancer diagnosis and treatment in the era of personalized medicine. Including sex into experimental design helps achieve responsible, rigorous and reproducible science (54). If incorporating both sexes in the research design is not possible, this should be indicated in article titles and trial reports (47).

Taken together, there is a strong rationale and growing evidence that patient sex is an important variable and it is time for sex bias in basic research and clinical medicine to end, as it will improve therapy for both sexes. For guidance on how to include sex as a biological variable in your research we recommend the SAGER guidelines (48) and the Gendered Innovations Annex A from the European Commission Directorate (5). We will conclude with The European Society for Medical Oncology recommendation that “Men and women with nonsex-related cancers should be considered as biologically distinct groups of patients, for whom specific treatment approaches merit consideration” (55).

Funding and acknowledgements
JG was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia Grant agreement No. 451–03–47/2023–01/200043, by the Science Fund of the Republic of Serbia, PROMIS Grant No. 6056979, REPANCAN, and the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 891135. This review was based upon the work within the COST Action CA20137, supported by the European Cooperation in Science and Technology.

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