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A graphic illustration of double-stranded DNA

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EDITORIAL

The role of metalloproteinases in atherosclerosis

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Metalloproteinases are multidomain zinc-dependent endopeptidases, also termed metzincins, that are involved in extracellular matrix (ECM) turnover, proteolysis of collagen, elastin and other ECM proteins, and several pathological pathways such as chronic inflammation.[1,2] Atherosclerosis is one of the most important causes of cardiovascular disease and is strictly related to inflammatory processes and protease activity.[3,4] The occurrence of atherosclerotic plaque rupture may dictate, for example, events such as myocardial infarction and stroke that are the primary contributors to disability and mortality worldwide.[5] Furthermore, plaque rupture is mainly caused by the metalloproteinases that play a role in regulating the content of ECM proteins in the fibrous cap and in the near regions of the necrotic core of plaques.[3] MPs are also able to influence all aspects of pathophysiology of atherosclerotic phenomena not only through their proteolytic activity but also through their actions on endothelial cells, vascular smooth muscle cells (VSMCs), and macrophages.[1-3]

There are three main families of metalloproteinases, namely, matrix metalloproteinase (MMP), which is the first identified and most widely known family; a disintegrin and a metalloproteinase (ADAM); and a disintegrin and a metalloproteinase with thrombospondin motif (ADAMTS).[1,2] Metalloproteinases are physiologically inhibited by tissue inhibitors of metalloproteinases (TIMPs) and help maintain ECM homeostasis.[1,2]

Endothelial cells, VSMCs, and macrophages are cellular source of MMPs. In particular, overexpression of MMP-1, MMP-8, and MMP-13 has been found in unstable plaques. Elevated MMP-2 plasma levels have been detected in coronary artery disease (CAD) patients. Increased MMP-9 expression and activity is related to the recruitment of several proinflammatory cytokines, both in coronary unstable plaques of CAD patients, and in carotid unstable plaques of carotid artery stenosis (CAS) patients. MMP-9 is often used as a biomarker in several clinical scenarios in patients with atherosclerotic diseases.[1,2] There is sufficient evidence from the current troves of research that targeting MMP could have therapeutic effects in patients with atherosclerotic diseases.[1,2]

ADAM members play an active role in the recruitment of inflammatory cells influencing atherosclerosis onset, progression, and complications, but, to date, there are no conclusive data on whether targeting these molecules may have therapeutic effects in patients with atherosclerosis.[1-3]

ADAMTS family is largely involved in atherosclerosis mainly for their ability to degrade proteoglycans which compose early atherosclerotic lesions with intimal thickening, and, in particular, ADAMTS-4 and ADAMTS-8 are also able to recruit monocyte/macrophage population triggering chronic inflammation within the artery. Genome-wide association studies (GWASs) have found single-nucleotide polymorphisms within the genomic region of *ADAMTS7*, which are associated with CAD. Histologically, high expression of ADAMTS-7 has been detected in plaques related to CAD and CAS. In fact, ADAMTS-7 seems to play a key role in promoting VSMCs migration and neointima formation.[3]

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There are only four members of TIMP family: currently, there is no evidence of specific roles for TIMP-1, TIMP-2, and TIMP-4 in atherosclerosis, whereas TIMP-3 was found to play protective roles in positively influencing plaque stability and effectively counteracting metalloproteinase activity.[3]

Over time, several synthetic MMP inhibitors have been identified with the aim to prevent or to treat clinical manifestations related to atherosclerosis, but the results obtained have been far from encouraging. Despite that, MMPs hold promise as potential biomarkers for several diseases related to atherosclerosis such as CAD, CAS, peripheral artery disease, and aneurysms.

Novel research unraveling the genomics, transcriptomics, and proteomics of metalloproteinases is warranted to clarify their roles in disease development and progression so that safe and effective strategies can be formulated to prevent and target clinical events associated with atherosclerotic disease.

Conflict of interest

The author declares no conflicts of interest.

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Gene & Protein in Disease

REVIEW ARTICLE

Advancing CRISPR technologies in reproductive biology

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) technology, a transformative tool for genetic modifications, gene therapy, and treating various genetic diseases, has recently garnered significant attention for its applications in reproductive biology. In this realm, researchers are focusing on addressing gynecological disorders, refining assisted reproductive methods, and conducting precise germ cell editing. The potential impact of CRISPR in these areas is monumental; however, several research gaps persist, demanding further investigation into the long-term effects, safety implications, and unintended consequences of its applications in reproductive biology. Refining the precision and specificity of CRISPR technology is a critical aspect that necessitates addressing challenges such as off-target effects and cytotoxicity. These considerations underscore the urgency for ongoing research efforts to ensure the efficacy and safety of CRISPR applications. In addition, the establishment of robust regulatory frameworks and oversight mechanisms specific to CRISPR in reproductive contexts is imperative to guarantee the responsible and ethical use of this powerful technology. This comprehensive review delves into the advantages of CRISPR over traditional gene editing methods, providing insights into its applications in embryos, pluripotent stem cells, and germline cells. It further explores the risks and limitations associated with CRISPR, including ethical concerns related to designer babies and eugenics. The article sheds light on the regulatory landscape governing new CRISPR applications in reproductive biology, emphasizing the continuous improvements in the efficiency and specificity of CRISPR technology. In contributing to the ongoing discourse, this review aims to inform and guide the responsible and effective application of CRISPR in the dynamic field of reproductive biology.

Keywords: CRISPR; Reproduction; Germ line editing; Gynecological disorders; Ethical concerns

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1. Introduction

The genome serves as the fundamental code governing the transmission of traits in living organisms and is the medium specifying hereditary patterns. Mammalian bodies, including humans, consist of trillions of cells, classified into germ cells known as gametes and autosomal cells called somatic cells.[1] Germline cells contain a single set of chromosomes, constituting a complete genome, while somatic cells typically possess two sets of chromosomes, representing two genomes.[2]

Scientists and researchers now have the privilege of accessing the complete sequence of the human genome, opening avenues for promising opportunities in genome modification for enhancement and therapeutic benefits.[3] This capability, known as gene editing or genetic engineering, involves making changes to nucleotide sequences in DNA, inducing artificial mutations through the insertion, deletion, or replacement of nucleotide bases.[4] Three prominent gene editing techniques – transcription activator-like effector nucleases (TALENS), zinc finger nuclease (ZFN), and clustered regularly interspaced short palindromic repeats–associated systems (CRISPR-Cas) – are currently in use, with CRISPR-Cas emerging as the most efficient for genome editing.[5]

Discovered in 1987 in *Escherichia coli*, clustered regularly interspaced short palindromic repeats (CRISPR) functions as part of the immune systems in prokaryotes against viral attacks.[6,7] Today, CRISPR has diverse applications, including gene therapy to eliminate genetic diseases, cancer treatment, and addressing mitochondrial diseases.[8-10] It has been successfully used to edit specific genes, such as *HBB,* in human tripronuclear zygotes.[11] Recently, the United Kingdom approved Casgevy, a CRISPR/Cas9 therapy by Vertex and CRISPR Therapeutics, for sickle-cell disease and β-thalassemia, demonstrating promising results in trials.[12,13]

Reproductive biology research contributes significantly to human welfare by advancing diagnostic methods and treatment plans for conditions such as endometriosis, preeclampsia, and infertility.[14] Understanding reproductive processes facilitate the development of innovative contraceptive options and improvements in assisted reproductive technologies (ARTs), benefiting couples facing infertility.[15,16]

CRISPR emerges as the pivotal component within gene editing technology, characterized by a single sequence length of 25 – 50 base pairs that are iteratively repeated. It has been effectively employed to treat various genetic diseases, including mitochondrial abnormalities, sickle cell disease, and cancer. Utilizing CRISPR in advanced reproductive biology holds the potential for CRISPR-based genetic engineering of animal models, gene therapy for heritable diseases, and assisted reproductive techniques. The review aims to provide an in-depth study of ongoing research, challenges, limitations, and future prospects in this field. We explore the applications of CRISPR-based genetic engineering in reproductive biology, discussing potential applications such as creating genetically modified animal models, gene therapy for reproductive diseases, manipulation of gametes and embryos, and assisted reproduction techniques. The review provides a comprehensive analysis of the current state of research in this field, highlighting benefits, challenges, and ethical considerations associated with the use of CRISPR in reproductive biology.

2. CRISPR technology

2.1. Basics of CRISPR

CRISPR, identified in the DNA of archaea and bacteria, serves as a defense mechanism against foreign DNA, such as bacteriophages, effectively neutralizing any undesirable effects of these foreign agents and operating as an immune response.[17,18] Japanese researchers first discovered CRISPR in 1987 during the study of the bacterial immune system at Osaka University.[19] The initial evidence of their existence stemmed from the identification of a distinctive repetitive DNA sequence in the *E. coli* genome, later designated as CRISPR. Subsequent discoveries revealed comparable sequence patterns in various bacteria, including halophilic archaea, indicating the evolutionarily conserved nature of these clusters of repetitive sequences for a crucial purpose. The connection between CRISPR and Cas proteins, initially believed to be involved in DNA repair in hyperthermophilic archaea, marked a significant step toward understanding the functional aspects of CRISPR-Cas systems.[7]

The gene-editing potential of CRISPR was initially reported in 2012 when Jennifer Doudna and Emmanuelle Charpentier outlined how to employ CRISPR to modify genes in human cells.[20] Their pioneering work on CRISPR earned them the Nobel Prize in Chemistry in 2020.[19,21-23] Since its inception, CRISPR technology has seen improvements in potency, cost-effectiveness, and efficacy, making it a valuable tool for diagnosing, preventing, and treating diseases.[21] In addition, novel nucleases like Cpf1 have been integrated into the CRISPR system, demonstrating enhanced efficiency compared to Cas9 and the ability to target multiple loci in the genome with a single crRNA transcript.[21,24] Researchers have developed innovative methods for gene editing at multiple loci while utilizing the same guide RNA (gRNA).[24,25] In today's technologically

advancing scientific landscape, Cas9–RNA complexes can be effectively employed as genome engineering agents in eukaryotic cells, encompassing those of plants and animals.[26-29] On the other hand, base editing through CRISPR enables precise DNA modification without causing double-strand breaks. In this regard, Xie *et al.*[30] efficiently induced C-to-T conversions at multiple loci, producing pigs with single or multiple point mutations in genes. Base Editor-Targeted and Template-free Expression Regulation (BETTER), which utilizes CRISPR-guided base editors for diverse multigene expression without library construction, has shown promise and is thus useful for large-scale refinement of expressions of multiple genes.[31] In addition, Li *et al.*[32] demonstrated a one-step approach for generating base-mutant mice using a third-generation base-editing system. The development of new base editor variants has expanded design possibilities, allowing recognition of various protospacer-adjacent motifs. For instance, Rosello *et al.*[33] outlined experimental strategies for cytosine base editing in zebrafish, allowing precise substitution of interest. Moreover, co-selection with loss-of-function mutations facilitates direct analysis of injected embryos, revealing the phenotypic impact of targeted substitutions. Conclusively, current progress in CRISPR gene editing and base editing suggests ongoing advancements in enhancing specificity, delivery, and ethical considerations for broader applications in disease management.

2.2. Components and mechanism of CRISPR

The CRISPR and associated systems are categorized into two main classes, encompassing six significant types with 33 subtypes and additional variants.[34,35] Class 1 comprises type I, type III, and type IV, while Class 2 includes the more prevalent type II, type IV, and type V.[35] Among these types, type II stands out as the most commonly utilized for genome editing.[36] Notably, type I and type II operate on the proteins Cas3 and Cas9, respectively.[37] The Cas9 system derived from *Streptococcus pyogenes* is the preferred choice due to its convenience and superior efficacy among all CRISPR-associated systems.[38]

The fundamental principle of CRISPR and associated systems is elucidated through Type II CRISPR technology. In Type II, the CRISPR system comprises two main components: A gRNA, primarily consisting of a 20-nucleotide sequence (crRNA) containing the target sequence complementary to one strand of the target DNA; and the transactivating crRNA that binds to the endonuclease Cas9 protein. The Cas9 endonuclease, a sizable protein, induces double-strand breaks (DSBs) at a specific site in the DNA. The CAS9–RNA complex encompasses two nuclease domains: the His-Asn-His domain, which cleaves the DNA strand complementary to the crRNA; and the RuvC-like Cas9 domain, which breaks down the non-complementary strand of the target DNA.[37,39,40] This process induces DSBs at the targeted DNA site.[41] Following the cleavage and editing of the targeted gene sequence, the DNA repair mechanism is activated, as illustrated in Figure 1. Two common repair methods are homology-directed repair (HDR) and non-homologous end joining (NHEJ). While NHEJ is more prevalent in mammals for DSB repair, it is less effective than HDR and may introduce unintended mutations. NHEJ simply ligates the two DNA strands with minimal processing. HDR is favored in Cas9-involved gene editing due to its high efficiency in repairing DSBs.[42-44]

2.3. Advantages of CRISPR over traditional gene editing technologies

CRISPR technology has surpassed traditional gene editing tools such as TALENS and ZFN in numerous aspects, presenting significant advantages. One such advantage lies in the high specificity of CRISPR technology for genome editing.[45] Continuous technological advancements further contribute to the reduction of off-target effects, with recent innovations such as prime editing demonstrating even greater precision and fewer off-site effects.[46] Notably, the simplicity of creation and implementation sets CRISPR apart from other gene editing technologies.[45] Its versatility and flexibility make CRISPR suitable for various genome-related studies, encompassing gene knockin and knockout, regulation of gene expression, and the management of hereditary conditions.[46] Moreover, CRISPR facilitates more effective location-specific gene editing, addressing challenges posed by classical gene therapy.[47] The capability of CRISPR to simultaneously activate multiple genes is a key advantage, overcoming limitations inherent in traditional gene overexpression technologies by incorporating numerous gRNAs in a single vector.[45] Conclusively, CRISPR technology offers numerous advantages compared to its predecessors, emerging as a pivotal tool in gene editing with reduced off-site effects and effective applications in gene therapy.

3. Applications of CRISPR in reproductive biology

The genetic revolution, coupled with advancements in genetic editing and genome engineering techniques over the past 40 years, has bestowed on us a profound understanding of molecular mechanisms and the complete sequencing of our genomes.[48] This transformative era has particularly empowered the application of gene-editing technologies like CRISPR/Cas9 in the realm of reproduction, a physiological phenomenon that stands as a prime target for intervention.

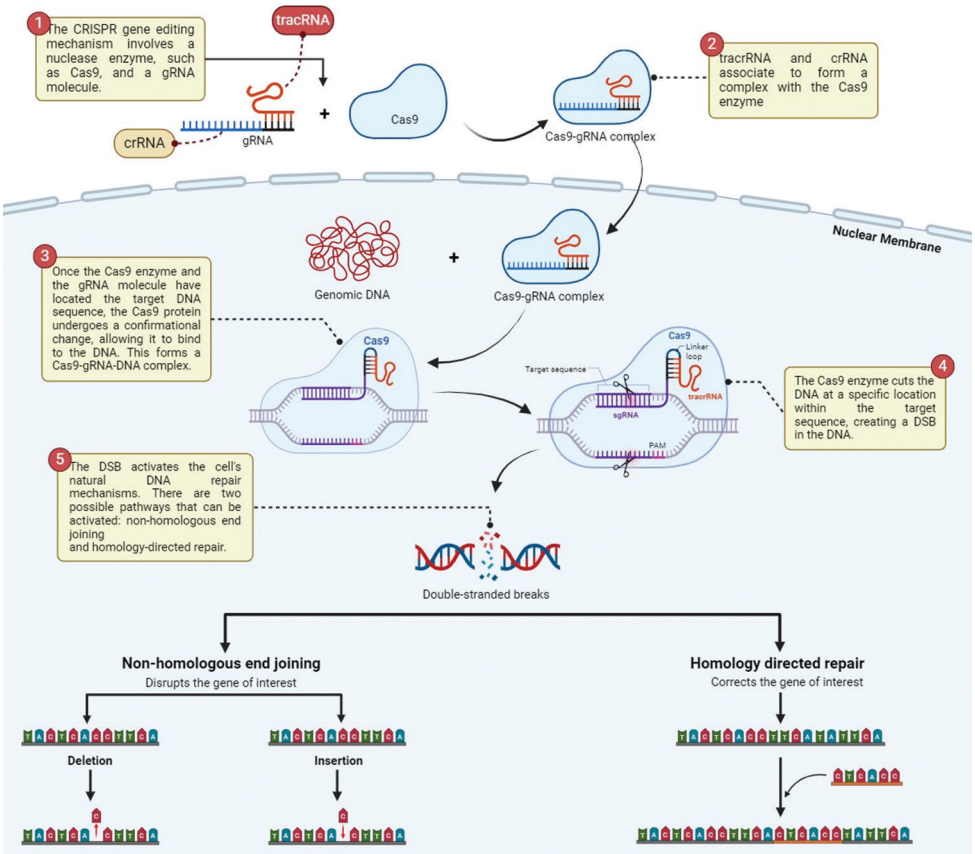

Figure 1. The CRISPR/Cas9 mechanism. It involves the combination of guide RNA (gRNA) with the Cas9 protein to form the gRNA–Cas9 complex. Following its formation, this complex is transported into the nucleus, where it interacts with the genomic DNA, creating the gRNA–Cas9–DNA complex. The His-Asn-His (HNH) and RuvC domains within Cas9 play a pivotal role in inducing double-strand breaks (DSBs) in the DNA. Subsequently, the DSBs triggered by the HNH and RuvC domains undergo repair through DNA repair mechanisms, specifically non-homologous end joining (NHEJ) and homology-directed repair (HDR). During this repair process, the DNA undergoes modifications in accordance with the desired changes initially introduced by the gRNA–Cas9 complex.
Abbreviation: CRISPR: Clustered regularly interspaced short palindromic repeats.

CRISPR technology, with its capability to precisely edit genes, has found extensive applications in correcting disease-causing mutations in embryos and enhancing the genetic health of individuals by addressing malformities in gametes. The potential of CRISPR in reproductive biology extends to the correction of a wide range of lethal and harmful ailments in living organisms.[49] For instance, CRISPR/Cas9 has been instrumental in creating a sperm-marking variant of the invasive fruit pest *Drosophila suzukii*, offering valuable insights for surveillance and reproductive biology studies.[50]

Advancements in microfluidic systems have further contributed to the study of female reproductive biology, holding promise for the development of more effective ARTs and therapies for conditions exclusive to women.[51] In essence, CRISPR-based genetic engineering serves a multifaceted role in gene therapy,[44,52] genetic editing in germ cells and embryos, correction of reproductive disorders, advancement of ARTs, generation of animal models for biomedical research,[53] and even holds potential for organ transplants. The breadth of applications underscores the transformative impact of CRISPR technology in the domain of reproductive biology.

3.1. CRISPR-mediated genome editing in germ cells

Germ cells, comprising haploid cells with half the chromosome number of somatic cells, play a pivotal role in reproduction. During fertilization, these cells, namely, sperm and egg, combine to form a zygote, initiating the development of an embryo and ultimately giving rise to an adult organism.[54] However, the application of genome editing technologies, such as CRISPR, to germ cells introduces the potential for transmitting genetic mutations to subsequent generations. This transmission of genetic mutations to future populations through germ cells can have significant consequences, impacting a large

number of individuals over a relatively short period.[55] A specific illustration of such consequences is observed in males experiencing infertility due to a mutation in the *AKAP3* gene, as depicted in Figure 2. This mutation disrupts the proper functioning of AKAP3, a protein crucial for anchoring protein kinase A in processes like capacitation and hyperactivated motility.[56] In addressing this challenge, CRISPR technology emerges as a promising solution. By leveraging CRISPR, it becomes feasible to correct the genetic mutation in the *AKAP3* gene, offering a potential avenue for the treatment of male infertility resulting from such genetic mutations. This utilization of CRISPR underscores the transformative potential of CRISPR in mitigating hereditary reproductive disorders and advancing therapeutic interventions.

One of the most impactful instances of human germline genome editing was claimed by Chinese scientist Jiankui He. He asserted that he successfully modified the genomes of embryos using CRISPR technology, resulting in the altered *CCR5* gene in a pair of newly born baby girls – a gene crucial for recognizing human immunodeficiency virus (HIV) and rendering individuals susceptible to the condition. The claimed purpose of editing the gene was to protect the babies from HIV transmission, as the father was HIV positive. However, subsequent studies revealed potential adverse effects of *CCR5* mutations, including increased susceptibility to lethal infectious diseases like influenza and a heightened risk of severe sclerosis, potentially leading to premature death.[55,57]

In a 2015 study, scientists utilized human tripronuclear zygotes (3PN) at an early stage for CRISPR-based gene editing, specifically targeting the human endogenous β-globin gene. While the efficiency of CRISPR in cleaving and modifying the targeted gene was demonstrated, challenges such as mosaicism in the embryo and off-target mutations were identified. Overcoming these challenges is

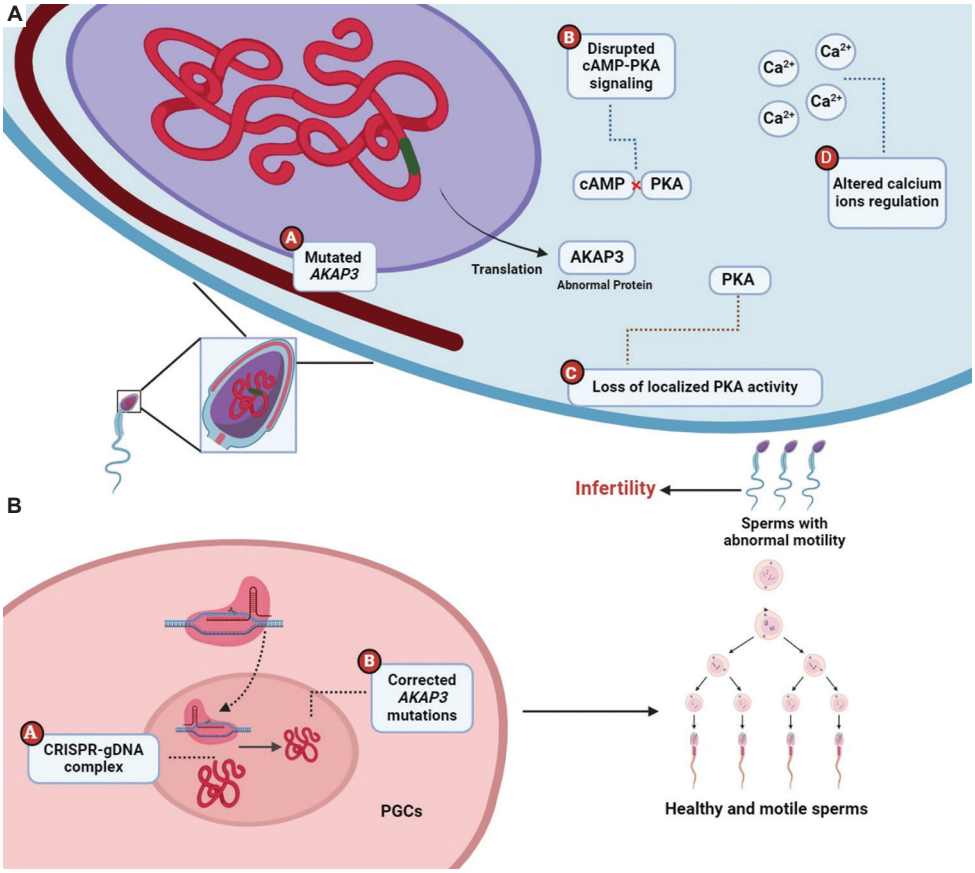

Figure 2. Genetic mutation in the gene *AKAP3* leads to infertility in the males. (A) Genetic mutation in the *AKAP3* gene disrupts its function in anchoring protein kinase A (PKA) (for capitation and hyperactivated motility), leading to abnormal protein. This disruption impairs localized phosphorylation of flagellar proteins, compromising coordinated flagellar beating and reducing sperm motility. cAMP and calcium are also crucial. cAMP activates PKA, regulating flagellar proteins' phosphorylation. Calcium ions guide microtubule sliding, shaping the flagellar movement. Disruption due to *AKAP3* mutation hampers these signals, reducing sperm motility. (B) CRISPR can be utilized to correct the genetic mutation, thereby serving as a potential agent to cure male infertility arising due to genetic mutations.
Abbreviations: PGCs: Primordial germ cells; CRISPR: Clustered regularly interspaced short palindromic repeats.

crucial for harnessing the full potential of CRISPR to modify genomes according to desired outcomes.[11] Furthermore, it has been shown that the CRISPR/Cas system can induce mutations in mature oocytes during meiosis, offering a potential avenue to alter specific fertility-related genes or prevent genetic defects in children.[58] However, ethical concerns, along with biological and physiological considerations, have led most scientists to declare CRISPR editing of germline genomes as currently unethical on clinical scales. Nevertheless, research activities persist, aiming to find safe and sound solutions to overcome these hurdles.[59]

3.2. Gene editing in embryos

The application of CRISPR on human embryos holds the potential to completely eliminate genetic abnormalities from the genome.[60] Utilizing CRISPR/Cas9 for modifying embryos, germline cells, and pluripotent stem cells in human reproduction exhibits significant promise.[61] However, the use of CRISPR gene editing to cure diseases in embryos is a subject of controversy. While CRISPR/Cas9 is extensively employed in scientific research, utilizing germline genome editing in clinics raises ethical and social concerns regarding the safety of future generations and the potential misuse of genome editing for human enhancement.[62] Despite these ethical considerations, studies have demonstrated the preventive and curative potential of CRISPR-based gene editing technologies for diseases caused by genetic mutations.[63] Notably, CRISPR-Cas9 has been successfully used to delete faulty genes associated with Parkinson's disease both *in vitro* and *in vivo*. In a rat model of Parkinson's disease, CRISPR-Cas9 effectively restored normal cellular functions and alleviated Parkinson's motor symptoms, suggesting its potential application in treating diseases caused by specific mutations, such as the A53T-*SNCA* mutation linked to Parkinson's disease.[64]

In addition, CRISPR gene editing aids in identifying damaging mutations in genetic diseases. For example, CRISPR/Cas9 was employed to create Lynch syndrome-related missense variants in the *MSH2* gene, a disorder induced by germline DNA mismatch repair gene mutations. The impact of these variants on cellular function was examined, showcasing the potential of CRISPR in understanding and addressing genetic disorders.[65] While concerns and moral ramifications surround the use of CRISPR gene editing on human embryos, ongoing studies explore the potential and limitations of CRISPR gene editing in embryos for various applications, including the development of large-animal research models and the treatment of genetic and reproductive problems.[66-68] Further, research is needed to address ethical, safety, and efficacy considerations before widespread clinical applications.

3.3. Gene editing for assisted reproduction techniques

ARTs represent medical procedures primarily employed to address reproductive disorders in individuals, facilitating the conception of babies. Examples of these techniques include donor insemination, egg donation, intracytoplasmic sperm injection (ICSI), and *in vitro* fertilization (IVF).[69-71] The increasing prevalence of these technologies has raised concerns about their safety, with reported complications such as low birth weight, preeclampsia, limited epigenetic variability, compromised embryonic quality, and stress.[72,73]

To address these challenges, CRISPR/Cas9 gene editing techniques have been proposed as a means to achieve precise and targeted genome modifications, potentially advancing various aspects of reproduction.[74] For instance, gene editing holds promise in treating fertility issues such as tubal disease and low sperm counts.[75] In addition to these applications, the combined use of CRISPR and ARTs has streamlined the editing of genomes in embryos produced through techniques like IVF. CRISPR/Cas proves particularly valuable in the context of IVF. Embryos produced through IVF can undergo CRISPR-mediated disruption or editing of specific genes, presenting opportunities to prevent genetic illnesses or enhance specific traits.[76] Furthermore, CRISPR holds the potential to enhance oocyte and sperm fertility within the IVF setting. For instance, the CRISPR/dCas9 activation approach has demonstrated success in restoring oocyte fertility by augmenting the levels of the sperm-oocyte binding protein Juno.[58] Overall, CRISPR technology has the potential to enhance IVF outcomes and foster the exploration of novel applications for both female and male reproductive systems.[77]

The integration of CRISPR technology with ICSI has been instrumental in several investigations focusing on male fertility and spermatogenesis. CRISPR/Cas9, for instance, was utilized to create *Tbc1d21* knockout mice, which exhibited male sterility due to bent spermatozoa flagella. While IVF proved ineffective for these mice, ICSI resulted in the birth of healthy offspring.[78] In another study, CRISPR/Cas9 was employed to generate *Iqcn*-knockout mice, which exhibited abnormal acrosome structures leading to male sterility. The failure of fertilization associated with *Iqcn* disruption was successfully managed using ICSI and aided oocyte activation.[79] These findings underscore the potential of CRISPR technology combined with ICSI to explore the genetic basis of male infertility and develop viable treatments.[80]

3.4. Producing genetically modified animals for research

In the rapidly advancing medical and pharmaceutical landscape, a substantial gap exists between the potential of gene editing technologies and their practical application in clinical trials. However, CRISPR/Cas9 facilitates the generation of model organisms, bridging the divide between proof-of-concept studies in small animals such as rodents and human clinical trials.[81] In biomedical research, a variety of animals, including zebrafish, mice, rats, monkeys, frogs, rabbits, dogs, pigs, and cats, serve as experimental models.[82-85] Non-human primate (NHP) models, due to their strong resemblance to humans, are particularly advantageous and can be generated by genetically altering NHP zygotes.[82]

Researchers have developed protocols for assisted reproduction and genetic alteration in NHPs, incorporating techniques such as ICSI, ovarian stimulation, *in vitro* oocyte maturation, IVF, embryo culture, and embryo transfer. Genetic engineering methods, including gene knockout and knockin techniques using gene editing protocols, have been employed alongside emerging gene editing methods for creating genetically modified NHP models for biomedical research.[71]

In a recent study aiming to create human models of liver cancer, researchers utilized the CRISPR/Cas9 system to induce loss-of-function mutations in *PTEN* and *P53* genes in monkeys. CRISPR demonstrated significant promise in generating tissue models of human liver cancer, aiding in drug discovery and development.[86] Similarly, monkeys with biallelic mutations of *P53* were produced using CRISPR/Cas9, closely mimicking genetic malfunctions in humans and potentially offering avenues for curing human genetic disorders.[87] In addition, animal models generated through CRISPR/Cas9, such as *Dnajb7* knockout mice, revealed no defects in reproductive health, indicating that *Dnajb7* is not necessary for mouse fertility.[88]

3.5. Potential for gene therapy in reproductive disorders

CRISPR technology holds significant promise for enhancing reproductive health in various ways. It can be employed to rectify harmful genetic and reproductive disorders, ultimately contributing to improved reproductive health and the prevention of certain genetic and reproductive issues (Figure 3).[49] CRISPR gene therapy has demonstrated efficacy in treating diverse diseases and genetic problems,

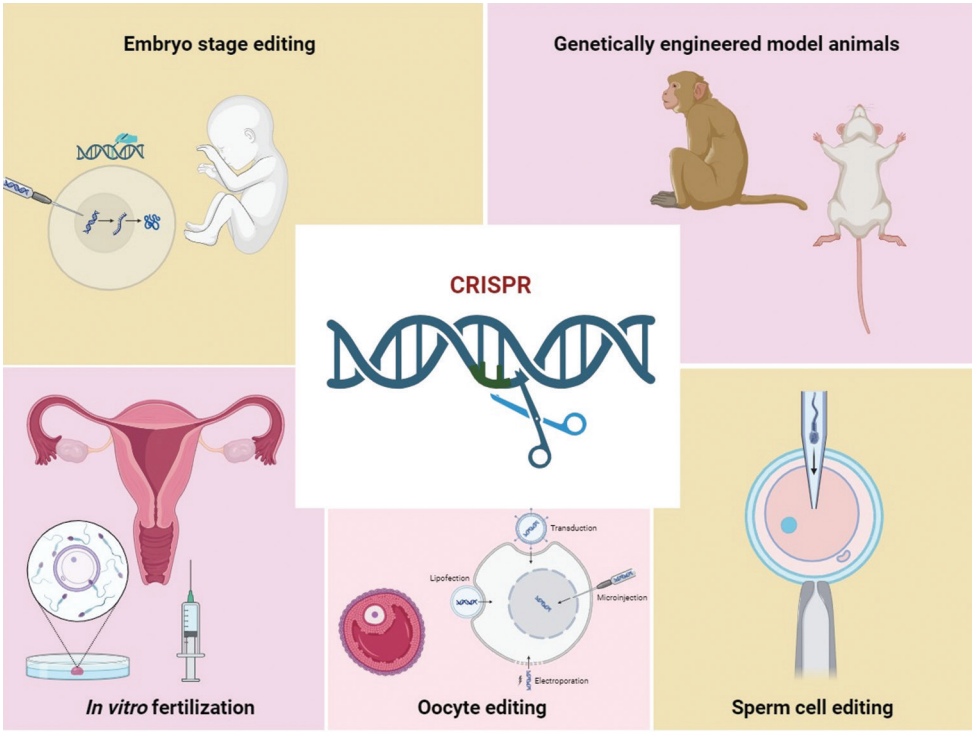

Figure 3. The use of CRISPR technology in treating various assisted reproductive methods. CRISPR is a gene editing technology used as a therapeutic approach for mitigating a wide array of reproductive defects and enhancing assisted reproductive methods. Specifically, CRISPR exhibits promise in treating genetic defects present in embryos, oocytes, and sperm cells. Moreover, it holds the potential to ameliorate malfunctions encountered in *in vitro* fertilization procedures. Furthermore, CRISPR facilitates the creation of genetically altered animal models for various experimental purposes.
Abbreviation: CRISPR: Clustered regularly interspaced short palindromic repeats.

with a particularly promising application in *in vivo* treatment for genetic disorders in humans.[89] Gene therapy shows potential in addressing gynecological disorders, including ovarian, cervical, and endometrial cancer, uterine leiomyomas, endometriosis, and complications with placental and embryo implantation.[90] Research on premature ovarian insufficiency explores genetic therapy targets such as the follicle-stimulating hormone receptor, apoptosis management alterations, Sal-like four gene polymorphisms, and deficiencies in thymulin or basonuclin-1.[91]

CRISPR research extends to comprehending and treating various diseases, including reproductive system cancers. A synthetic technique for *in vivo* CRISPR-mediated activation of tumor suppressor genes has shown promise in reducing tumor burden.[92] CRISPR-Cas9 gene editing exhibits potential in treating gynecological disorders such as cervical, ovarian, and endometrial cancer.[93] Studies on endometrial cancer reveal the preventive effects of cationic microbubbles containing paclitaxel and CRISPR/Cas9 plasmids in a mouse xenograft model.[94] Animal models of endometrioid carcinoma and high-grade serous carcinoma have been developed using CRISPR/Cas9-mediated somatic gene editing.[95] Despite the effectiveness of CRISPR-Cas9 in treating gynecological malignancies, further research is needed to address potential drawbacks, such as off-target effects, before widespread clinical application.[96] Although gene therapy for reproductive disorders is in its early stages, promising results suggest its potential role in future treatments.[90,91]

In conclusion, CRISPR technology holds promise for enhancing both male and female reproductive health, as evidenced by trials on NHPs and other animal models. However, ethical considerations must be carefully addressed in the application of this technology.

4. Risks and limitations of CRISPR technology

Despite the apparent advantages of CRISPR/Cas technology, several downsides require resolution. Particularly, its application in human embryos raises medical and ethical concerns due to the potential unforeseen consequences, such as off-target mutations, which may be inherited by subsequent generations.[97] Off-target effects, characterized by the unintended cleavage of DNA sequences similar to the target sequence, pose a significant challenge.[98,99] Another limitation is the low efficiency of multigene editing, which concerns the scientific community.[100] In addition, the CRISPR/Cas system is associated with cytotoxicity and immunotoxicity.[101] Moreover, the delivery of CRISPR components to the desired site is constrained, with methods such as viral vectors, DNA plasmid vectors, electroporation, and microinjections, each having drawbacks ranging from cytotoxicity to the permanent permeabilization of plasma membranes.[47]

4.1. Concerns over designer babies and eugenics

Genome editing in germlines holds the potential to eliminate heritable genetic disorders and enhance human health significantly. However, along with these potential benefits, there are accompanying risks and disadvantages, particularly if not used and regulated properly. A major concern is the concept of "designer babies",[57] where the use of genome editing in human embryos may usher in a new era of "new eugenics," potentially widening social disparities.[102] The contentious issue of creating genetically modified children using CRISPR technology raises ethical questions about the manipulation of infants, commonly referred to as designer babies.[103] Bioethical and social concerns surrounding genome editing in germline cells primarily stem from these ethical dilemmas. Another significant concern involves the potential application of CRISPR technology in eugenics,[104] where it could be employed to alter human attributes to create "superior" individuals or eliminate "undesirable" traits. This application raises ethical concerns due to the possibility of unforeseen consequences, discrimination, and the loss of genetic diversity.[105-107]

4.2. Regulation and oversight of CRISPR applications in reproductive biology

Regulations and monitoring mechanisms for CRISPR applications in reproductive biology are currently being developed by national and international regulatory bodies. These guidelines aim to address safety criteria, as well as social and ethical concerns associated with germline genome editing for reproductive purposes.[108] Regulations governing the use of CRISPR in reproductive biology vary by country and are continually evolving. One notable concern is the potential for dual-use research, where CRISPR technology might be misapplied or intentionally developed for harmful purposes.[109]

Historically, both international and state legislation has restricted genome editing, largely due to ethical considerations. However, there has been notable progress in reconsidering these laws. A recent consensus statement issued after the International Summit on Gene Editing in Washington, D.C., emphasizes the discouragement of germline editing, stating that it should only be considered in cases where couples are affected by diseases involving a dominant disease-causing allele in a homozygous state or rare recessive homozygous mutations considered lethal.[110] There is a pressing need for a comprehensive and impartial

assessment of the current uses and potential misuse of CRISPR technology to inform appropriate regulations in reproductive technology.

5. Potential for new applications of CRISPR in reproductive biology

CRISPR-based gene drives represent an emerging technique with significant implications in reproductive biology. In particular, CRISPR gene drives are valuable for managing disease vectors and invasive species populations. For example, gene drives targeting female fertility reduction can effectively decrease mosquito populations that serve as vectors for diseases such as malaria. Advanced gene drives have been developed to minimize resistance while imposing a high reproductive load on laboratory-contained mosquito populations.[111] In addition, gene drive technology is being explored as an alternative to rodenticides for controlling invasive rodent populations, offering potential benefits for agriculture, food security, conservation, and human health.[112,113]

Another avenue in CRISPR gene editing for reproductive biology involves epigenetic modifications. CRISPR technology holds significant promise in contributing to the field of epigenetic modifications, offering avenues to alter genes, remodel germ lines, and manipulate sex ratios.[114] This precise gene editing technique, applicable both *in vitro* and *in vivo*, possesses broad biological and medical applications.[115] Through the targeting of genes involved in spermatogenesis and other reproductive processes, CRISPR can facilitate the investigation of epigenetic alterations in reproductive biology, thereby enriching our understanding of gene function and disease treatment.[80] Furthermore, CRISPR-mediated mutations can alter methylation patterns, providing insights into the epigenetic control of gene expression.[116] Overall, CRISPR technology emerges as a powerful tool for studying the epigenetic changes during reproductive processes and for developing new therapeutic approaches to address reproductive health issues.

Furthermore, CRISPR/Cas genome editing has revolutionized the field of reproductive biology, enabling the creation of knockout and knockin animals in novel ways that were previously challenging.[117] The development of efficient CRISPR tools for genome editing, coupled with rapid methods for their introduction into mammalian cells and mouse zygotes, has yielded significant benefits for the study of reproductive biology.[118] Another potential application in the near future is the development of contraceptives using CRISPR technologies. CRISPR has been utilized to study genes involved in crucial fertility processes in both male and female model organisms.[119] For instance, a study using CRISPR/Cas9 to generate *Tssk3* knockout mice identified TSSK3 as a crucial factor in spermiogenesis, suggesting its potential as a target for the development of non-hormonal male contraceptives.[120]

6. Improvements in efficiency and specificity of CRISPR technology

To enhance the specificity and efficiency of CRISPR systems and address associated challenges, researchers have pursued various strategies. One approach involves the modification of the Cas9 enzyme to enhance its selectivity, while another strategy focuses on the development of new software for designing improved gRNA.[121] Studies on the optimization and engineering of highly specific single gRNA (sgRNA) have demonstrated different techniques. For instance, one study improved sgRNA specificity by optimizing its length, thereby reducing the risk of off-target modifications.[122] Efforts to enhance the efficiency and specificity of CRISPR technology also include the design of smaller nucleases. In an *in vivo* study conducted on mouse liver, scientists synthesized small RNA-guided nucleases (sRGN), demonstrating high specificity and efficiency in both mouse liver and human cell lines. These synthetic RGNs were further explored as a delivery mechanism advantageously packed in an all-in-one adeno-associated virus vector.[123] In addition, nanoparticle-based delivery systems, such as lipid-based nanoparticles, have shown potential in increasing the specificity of CRISPR technology, particularly in achieving high tissue-specific gene editing in mouse lung and liver tissues.[124] To address off-target effects, modifications in off-target detection tests aim to reduce accidental cleavage, potentially enhancing the efficiency of CRISPR technology.[121,125] Another avenue involves the use of naturally occurring proteins, such as anti-CRISPR proteins like AcrIF1, AcrIF2, and AcrIF4, which can control and regulate CRISPR activities, confining gene editing to desired areas within the body.[84,126,127]

Despite the formidable capabilities of CRISPR, a significant research gap remains in accessing numerous biological targets, primarily due to the absence of efficient delivery systems following systemic injections. Notably, three distinct biomolecular Cas9 and gRNA formats, namely, plasmid DNA (pDNA), mRNA, and Cas9 ribonucleoproteins, pose challenges in this context. In a study by Shinmyo and Kawasaki,[128] pX330 plasmids expressing humanized Cas9 and sgRNAs were employed to target the *Satb2* gene, inducing precise mutations in the rodent brain through *in utero* electroporation. This approach not only allowed for precise gene manipulation but also provided insights into the functional consequences within neural development, offering promise in exploring

gene function in brain development. Advancements in CRISPR technology precision and effectiveness have ushered in a new era of genome editing capabilities. Co-delivery of Cas9 mRNA and sgRNA offers an efficient strategy, especially for editing non-dividing cells, with a focus on minimizing safety concerns. Addressing the challenge of co-delivering RNA molecules with distinct sizes, Abbasi *et al.*[129] utilized a PEGylated polyplex micelle (PM) to encapsulate both Cas9 mRNA and sgRNA, demonstrating widespread genome editing across various parenchymal cells in the mouse brain. Similarly, Chen *et al.*[125] reviewed the potential of synthetic carriers for achieving tissue-selective gene editing by co-delivering Cas9 mRNA and sgRNA targeting PCSK9 using selective organ-targeting lipid nanoparticles (LNPs).

Researchers have tackled the delivery challenge of Cas9 ribonucleoprotein (Cas9 protein plus sgRNA), which arises from the large size of Cas9 and the negative charge of sgRNA. Wei *et al.*[124] developed lung-targeting 5A2-DOT-50 LNPs, achieving tissue-specific gene editing in the livers and lungs of mice with high efficiency. Kai *et al.*[130] engineered a thermostable GeoCas9 variant for robust genome editing, demonstrating significant editing efficiency in the liver and lungs of mice. Shen *et al.*[131] constructed traceable nano-biohybrid complexes (F-TBIO) for efficient delivery of CRISPR/Cas9 plasmids into brain lesions, successfully knocking out the *Bace1* gene in mice with Alzheimer's disease. Lee *et al.*[132,133] developed CRISPR-Gold, a gold nanoparticle-based system for CRISPR delivery, demonstrating superior HDR efficiency, lower toxicity, and potential therapeutic applications across various contexts.

In summary, these strategies represent promising advancements in targeted Cas9 delivery using various systems, opening new avenues for enhanced genome editing. Future research may focus on refining delivery strategies, particularly for therapeutic applications, with a notable emphasis on reproductive systems.

7. Conclusion and future prospectives

The discovery of CRISPR technology presents significant potential for revolutionizing reproductive biology and addressing a broad spectrum of genetic and reproductive issues. This technology opens up novel avenues for treating gynecological tumors, reversing deleterious genetic changes, and advancing contraceptive development. Nonetheless, it is crucial to conscientiously address and regulate ethical concerns, encompassing the risks of unforeseen mutations, the ramifications of germline editing, and the ethical considerations surrounding eugenics and designer babies. CRISPR technology is poised to revolutionize research in reproductive biology, with potential breakthroughs in germplasm preservation, the generation of artificial gametes and gonads from stem cells, and further investigations into reproductive microbiomes.[134] The realization of CRISPR's full potential in editing germlines, gametes, and gametic precursors to benefit the human community relies on overcoming challenges such as off-target mutations, mosaicism, and ethical considerations. Ongoing enhancements in CRISPR systems, including the refinement of Cas9 enzymes, improved gRNA design, utilization of smaller nucleases, nanoparticle-based delivery techniques, and exploration of anti-CRISPR proteins, contribute to increasing efficacy and specificity. With continued research and judicious regulation, CRISPR technology holds the promise of significantly impacting reproductive health and contributing to the well-being of individuals and future generations.

Addressing ethical and safety concerns associated with the modification of CRISPR technology requires responsible usage and management in reproductive applications. Striking a balance between moral considerations and scientific advancements is essential. Comprehensive legislation must be enacted to address safety, social, and ethical issues, ensuring careful navigation of potential pitfalls. To minimize unexpected outcomes, efforts should be directed toward enhancing the precision and accuracy of CRISPR systems. Through fostering a collaborative and interdisciplinary approach, we can harness the benefits of CRISPR while upholding moral standards, safeguarding individual welfare, and ensuring the well-being of present and future generations.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Natural carotenoids as a potential chemopreventive agent for prostate cancer: A literature review

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Abstract

Prostate cancer (PCa) is the most commonly diagnosed cancer in men and the second leading cause of cancer death among men worldwide. While the exact etiology of PCa remains unclear, various factors contribute to the onset of the disease. These factors include modifiable risk factors such as physical activity, diet, obesity, smoking, alcohol consumption, and exposure to environmental agents. In addition, unmodifiable risk factors such as age and ethnicity play a role, with men of African ancestry being more susceptible to the disease. Despite the availability of potential treatment options, prevention is of utmost importance in reducing the incidence of PCa. Researchers have turned their attention to carotenoids, which are natural compounds derived from fruit and vegetables such as citrus, tomato, and green leafy vegetables, due to their potential chemopreventive effects. Multiple phase II clinical trials have indicated a reduced incidence and progression of diagnosed PCa in patients. Laboratory studies on PCa cell lines have demonstrated that carotenoids induce apoptosis and reduce cellular accumulation and adhesion of PCa cells in a dose-dependent manner. In this literature review, we assess the chemopreventive potential of the most common carotenoids: α-carotene, β-carotene, lycopene, lutein, and β-cryptoxanthin, which are often found in a heterogeneous mixture. We also discuss their potential clinical use as well as challenges related to their safety and bioavailability. Overall, a better understanding of the etiology and pathophysiology of PCa will lead to the development of improved preventative strategies and treatments for the disease.

Keywords: Prostate cancer; Chemoprevention; Carotenoids; Natural products; Antioxidant properties; Apoptotic properties

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1. Introduction

1.1. Overview of prostate cancer (PCa)

PCa stands out as the most widespread malignancy among males over the age of 45,[1] marked by elevated mortality rates worldwide. In 2020 alone, there were over

1.4 million newly reported cases and 375,000 recorded deaths.[2] Histologically, PCa arises from luminal epithelial cells characterized by heightened expression of androgen receptors (ARs) and differentiation antigens, including cytokeratin 8 and prostate-specific antigen. Basal cells, marked by a lower intensity in the expression of ARs, and sporadic neuroendocrine cells also play roles.[3] The development of prostate adenocarcinoma is attributed to the inactivation of tumor suppressor genes such as *SMAD4*, *PTEN*, and *TRP53*, which can occur either in both luminal and basal cells or independently in each cell type. It remains unclear if neuroendocrine cells found in the stratum basale (also known as basal layer)[4] play a role in prostate neoplasia. The transformation of cells derived from normal human prostate epithelial tissue into malignant cells results in the development of prostate adenocarcinoma, squamous carcinoma, or neuroendocrine carcinoma, with metastatic PCa being the primary contributor to mortality. Metastases commonly occur in bones, lungs, and liver.[5] Epidemiologically, PCa displays heterogeneity, with occurrence rates ranging from 6.3 to 83.4/100,000 individuals globally. Regions such as Southern Africa, Australia, the Caribbean, North America, and Western and Northern Europe exhibit the highest percentages of prostate malignancy, while North Africa and Asia exhibit the lowest rates. Mortality rates show notable variations compared to incidence rates, with the Caribbean recording the highest rates at 75.8/100,000 people, followed by sub-Saharan Africa at 22.0/100,000 people, and Micronesia/Polynesia at 18.8/100,000 people. Furthermore, it is widely acknowledged and documented that men of African ancestry have a higher predisposition to develop PCa during their lifetime, while men of Asian descent appear to be less susceptible.[6]

1.2. Etiology of PCa

The onset of PCa is influenced by a multitude of factors, both modifiable and unmodifiable, contributing to its multifactorial etiology. Particularly, factors contributing to susceptibility to PCa include aging, family history, genetic predisposition, and ethnicity.[6] As far as age is concerned, PCa is infrequent before the age of 40, with the average age at diagnosis being 66 in North America.[7] It is worth noting that the occurrence of prostate neoplasia rises with age, a pattern observed in both developed and developing countries. For men aged 38 and below, the likelihood of acquiring PCa is 0.005%.[8] In addition, PCa exhibits heightened heritability, with men having a two- to four-fold increased risk of developing the disease if their father or brother has been diagnosed with it. Studies have also demonstrated that families with traits of both familial breast cancer and familial PCa have an elevated risk of developing PCa. Family history involves a combination of genetic and epigenetic factors, which will be explored in the subsequent subsections. Ethnicity is another significant factor, with men of Black African ancestry experiencing a higher incidence, greater severity, and increased mortality rates in relation to PCa. Various factors, including smoking, alcohol consumption, obesity, physical exercise, diet, medications, and sex hormone-related factors, can influence the risk and progression of PCa.[6]

1.2.1. Genetic mechanisms

Translocations involving transcription factors of the E26 transformation-specific (ETS) family, such as *ERG* genes, and androgen-regulated promoters appear to be the most frequent genomic alterations in prostate carcinoma. The activation of ETS contributes to carcinogenesis through diverse mechanisms, encompassing lineage specification, epigenetic modifications, genome instability, and remodeling of metabolism. In addition, the amplification of the *MYC* gene enhances the progression of PCa by activating protumorigenic factors pivotal for cell growth and development through transcription. AR signaling plays a central role in prostate function and maturation. Thus, any mutation or amplification of AR causes the development of PCa. Furthermore, *PTEN* functions to inhibit the PI3K–AKT–mammalian target of the rapamycin (mTOR) pathway, thereby regulating cell survival, proliferation, and metabolic processes related to energy production. The loss of *PTEN*, often due to deletion and mutation, is estimated to occur in 40% of prostate neoplasia cases.

In addition, *SMAD4* serves as a tumor suppressor, exerting influence on downstream activity in the TGFβ pathway. It plays a vital role in regulating gene transcription, suppressing epithelial cell growth, and reshaping the tumor microenvironment (TME). TME refers to the normal cells and elements existing within the tumor, encompassing substances generated and released by them. Continuous interactions between cancerous cells and the microenvironment surrounding the tumor are crucial in the initiation, advancement, spread, and reaction to treatments of the tumor.[7]

BRCA genes are pivotal in transcription and DNA repair processes related to double-stranded breaks and recombination. Mutations in the germline of *BRCA* genes are linked to a heightened risk of PCa or a more invasive phenotype, resulting in a poorer prognosis. Finally, *SPOP* is a constituent of a BTB–CUL3–RBX1 E3 ubiquitin–protein ligase complex. Mutations in *SPOP* lead to the maintenance of tumorigenic molecules, including JNK, NCOA3, DEK, and BET family proteins.[5]

1.2.2. Epigenetic mechanisms

Epigenetics is involved in both the initiation and development of PCa. During tumor development, epigenetic

alterations in different genes and pathways, as well as modifications in methylation patterns, are observed. The glutathione-S-transferase P1 (*GSTP1*) gene has been implicated in prostate neoplasia, encoding an enzyme responsible for DNA protection against various factors, including carcinogens, and participating in the catalytic cycle of PRDX6, an important antioxidant enzym.[8] In prostate tumorigenesis, the *GSTP1* gene is not expressed in cancer cells since its promoter is methylated. The promoter region of *GSTP1* undergoes hypermethylation in around 75% of pre-invasive high-grade prostatic cancer and over 90% of prostate tumors. Hypermethylation also occurs at promoters of other genes involved in PCa, including adenomatous polyposis coli, Ras-associated domain family 1A, O6-methylguanine DNA methyltransferase, and others. In addition to DNA methylation, chromatin acetylation and/or histone modifications are also epigenetic alterations. In prostate carcinoma, the transcription process of AR effector genes is regulated by a cluster of transcription factors. Particularly, histone acetylation leads to the active transcription of target genes, as AR agonists conscript the AR and coactivators with histone acetyltransferase activity to the promoter of AR genes. On the contrary, gene expression is blocked by the connection of histone deacetylases with corepressors such as SMRT or NCoR, which are activated by AR antagonists.[5,9]

1.3. Risk factors

A variety of risk factors plays a crucial role in PCa tumor growth. PCa is a multifactorial disease, with numerous exogenous risk factors such as physical activity, diet, obesity, smoking, alcohol and environmental agents, and endogenous risk factors such as hormones, family history, race, and aging. While factors such as age and ethnicity are unalterable, others such as diet can be modified.[10]

1.3.1. Hormones

Recent studies have pointed out the complexity of the interaction between a wide range of hormones such as testosterone, free testosterone, sex hormone-binding globulin (SHBG), 5-alpha reductase, and estrogens, making it difficult to determine the hormones' precise role in prostate carcinogenesis. While multiple studies have reported elevated testosterone levels in cancerous tissue, these levels have not consistently correlated with aggressive disease.[11-13] Mendelian randomization (MR) analysis has suggested a correlation between high levels of free testosterone and an increased risk of aggressive PCa, although this association was not consistently observed in blood sample analyses. SHBG levels were not found to be associated with an elevated risk.[12] In addition, high concentrations of estrogens, whether from maternal exposure or pharmaceutical doses, have been implicated in stimulating tumorigenesis through prostate atrophy. Estrogens can initiate tumor growth as chemical carcinogens by activating metabolic pathways.[14] Patients prescribed 5-alpha reductase inhibitors for benign prostatic hyperplasia may face an increased risk of high-grade carcinogenesis.[15] However, other studies have suggested that the impact of 5-alpha reductase inhibitors on the risk of PCa is lower in Asian populations.[11] In addition to androgens and estrogens, insulin-like growth factor 1 (IGF-1), also known as somatomedin C, has an important impact on prostatic cancer. Numerous studies have investigated the correlation between elevated levels of IGF-1 and the risk of PCa.[16]

1.3.2. Family history

The association between family history and the incidence of PCa is well documented. Patients with this neoplasia can inherit the disease from relatives, and studies have established that a history of breast cancer in the family can also increase the likelihood of prostate carcinogenesis. Consequently, men with a family history of PCa should undergo more frequent screening.[11] The risk of PCa is influenced by factors such as the age of the relative at cancer detection and mortality, as well as the degree of family correlation.[17] Numerous studies have indicated that having a first-degree relative diagnosed with PCa may increase the risk by an estimated factor of 2.5. In addition, several studies assessing the age of relatives have found that younger men under 65 years of age in the family have a risk estimate of 4.3.[18] Hemminki and Czene's studies have highlighted the significance of the father's age at diagnosis, with an estimated risk of 3.55 for sons if the father was diagnosed before the age of 60, compared to a risk of 2.5 if the father was diagnosed after 60 years of age. Similarly, having an affected brother under the age of 55 is associated with a higher estimated risk of 8.05, whereas the risk decreases to 3.5 after the age of 55 years. In families where both the father and brother have been diagnosed with PCa, the risk for another son may be as high as 33.09 before the age of 55 years.[19] Moreover, PCa has been associated with familial cancer syndromes, such as hereditary breast and ovarian cancer syndrome (HBOC) and Lynch syndrome (LS). HBOC syndrome, characterized by mutations in *BRCA1* and *BRCA2* genes, is associated with multiple incidences of breast, ovarian, and pancreatic cancer in relatives. Studies suggest that men with hereditary mutations in *BRCA1* and *BRCA2* have a higher incidence and mortality rate for PCa. LS, involving mutations in the DNA mismatch repair system, increases the likelihood of PCa by threefold in affected men.[20]

1.3.3. Race

The incidence of PCa varies by geographic area. GLOBOCAN data indicate that the highest incidence is

observed in the United States and Europe, while the lowest incidence is found in Asia and Saharan Africa.[10,16,17] It is crucial to highlight the complexity of race and ancestry, as health-care facilities and socioeconomic factors play distinct roles in screening, rendering PCa diagnosis challenging in developing countries.[21,22] It has been pointed out that in certain regions, such as the United States, African–American groups exhibit an increased incidence (1.7 times higher estimated risk) than white people, owing to genetic factors,[10] with mortality rates being 2 – 3 times higher.[23] African–American men are twice as likely to develop the disease as Caucasian men and three times more likely than Asian men.[21] The higher prevalence among African-American men is likely related to genetic ancestry rather than race.[17] Differences in incidence rates can also be attributed to diet, environmental factors, culture, and habits, in addition to genetic background.[16] African–American men harbor a chromosome 8q24 variant, which is associated with PCa risk. Moreover, apoptosis gene *BCL2* and gene *EPHB2* are associated with the risk of prostate carcinogenesis.[22,23] Several studies have pointed out that prostate-specific antigen (PSA) levels are higher in American–African men without PCa than in other populations, and even higher than those of European men diagnosed with cancer.[24]

1.3.4. Aging

Men aged older than 65 are at a higher risk of being diagnosed with PCa.[10] Below the age of 65 years, the risk of PCa is lower, estimated to be under 30.7%. Therefore, age is positively correlated with the incidence of PCa in elderly men.[11] Moreover, mortality rates tend to increase with age.[24] In the United States, the estimated risk is 1.8% for individuals under the age of 60, 9% for those aged 60 – 69 years, and 12.5% for individuals over 70 years old.[17] Age is an important factor in determining the schedule of treatment. However, it is essential for men over 50 years of age to undergo annual examinations, including PSA tests or rectal examinations.[16]

1.3.5. Physical activity

While physical activity has been demonstrated to decrease the risk of several cancers, the clear association between physical activity and the incidence of PCa remains elusive. Individuals who are active in physical activity have been observed to have a lower risk of mortality from PCa compared to those who are sedentary.[25] In addition, the protective role of physical activity at work against this type of cancer remains uncertain.[26] Further research is needed to elucidate the role of physical activity in preventing PCa.

1.3.6. Obesity

Obesity, closely related to metabolic syndrome, significantly increases the risk of aggressive PCa, such as high-grade tumors with a Gleason score over seven.[27] Studies conducted on overweight individuals, defined as having body mass index (BMI) >25.0 in Asian countries and BMI >27.8 in the United States, have demonstrated a higher possibility of PCa incidence.[28-30] Chronic systemic inflammation in the body, attributed to obesity, further elevates the risk of prostate carcinogenesis. In addition, individuals with obesity exhibit low levels of free testosterone and luteinizing hormone, which are the factors associated with an increased risk of this malignancy.[31]

1.3.7. Diet and oxidative stress

Diet was first investigated as a significant risk factor by Muir *et al.*[32] Specific diets play a crucial role in PCa incidence. The Western diet, characterized by high consumption of meat products and fats, has been related to an increased risk of prostate carcinogenesis.[27,28] In contrast, the Mediterranean diet has demonstrated a beneficial impact on PCa risk.[17,27]

A high intake of overcooked red meat, fat, and dairy products has been associated with an increased incidence of PCa.[27,28] Specifically, high consumption of saturated fat has been related to cancer relapse, while unsaturated fatty acids, such as omega-6 fatty acids, are related to a high risk of incidence.[28,33] On the other hand, omega-3 fatty acids have been demonstrated to play a preventive role in PCa[28,34] by reducing levels of estradiol, testosterone, and androgens.[35] Moreover, diets high in fats are correlated with increased oxidative stress and inflammation.[27] Although red meat consumption alone has a weak association with PCa incidence,[36,37] cooking red meat at high temperatures can increase the risk of PCa due to the formation of mutagens, such as heterocyclic amines and polycyclic aromatic hydrocarbons.[35,37] Similarly, consuming dairy products has been identified as a risk factor for prostate carcinogenesis, particularly high consumption of milk and yogurt, which have been associated with high incidence and mortality rates in PCa.[17] This association may be attributed to the high concentration of calcium in dairy products, as increased calcium intake has been correlated with a high incidence of PCa.[38] While calcium is important for cell growth, high levels of calcium combined with low levels of vitamin D may induce tumorigenesis.[27]

1.3.8. Smoking and alcohol consumption

The role of smoking in the risk of PCa remains controversial. Tobacco contains over 4000 chemicals, with more than 60 identified as carcinogenic.[16] Smoking can cause DNA damage and increase the risk of various cancers, including PCa.[39] Furthermore, smoking contributes to the occurrence of metabolic syndrome, and high consumption of cigarette smoking can increase the risk of aggressive disease by 30%.[22] Smoking has an even more pronounced effect

on PCa, increasing the risk by 42%.[39] Asian individuals exhibit a polymorphism of the xeroderma pigmentosum complementary group C (*XPC*) gene, which is associated with nucleotide excision repair against DNA damage. Studies on the *XPC* gene have demonstrated that intron 9 (PAT) polymorphism is associated with a higher risk of PCa. Asian smokers with one or two alleles of PAT polymorphism have a higher incidence of this cancer.[40] Smoking can also increase the risk of mortality in PCa.[35] On the other hand, Pourmand *et al.* have demonstrated in their study that smoking does not affect the risk of PCa in Iranian people.[24] Male smokers exhibit increased levels of androgens, which may contribute to PCa risk.[16] The mechanism of smoking interaction in PCa progression is still controversial and requires further study.[27]

Alcohol consumption has been associated with an increased risk of various cancers in humans. Bergengren *et al.* briefly described the lack of correlation between alcohol consumption and the risk of PCa.[17] However, contrasting findings from other studies have demonstrated that alcohol consumption does pose a moderate, yet statistically significant, risk for PCa, particularly in relation to alcohol dose.[41] A review conducted by Perdana *et al.* indicates a dose-risk relationship, suggesting that consuming four drinks per day is associated with a higher risk of PCa, but moderate intake of red wine may have a protective impact.[16,21] Several factors contribute to the varying results regarding alcohol as a risk factor, such as the different types of alcohol consumed, dietary habits, and alcohol consumption history. Men with a long history of alcohol consumption over many years have a high incidence of developing PCa. In addition, men with a family history of PCa may need to exercise caution with alcohol use.[42]

1.3.9. Environmental agents

Except for nutritional and genetic risk factors, environmental agents must be considered in the risk factors for PCa. Studies have associated sunlight, trace minerals, farming, and synthetic hormones as potential risk factors for PCa. Farmers, in particular, exhibit a higher risk of incidence and mortality than other workers, possibly attributed to pesticide exposure, such as phorate, coumaphos, and butylate.[10,43] Butylate, coumaphos, and phorate have been associated with a high incidence of PCa, especially in men with a family history of this cancer, but not among men without a family history.[44-46] Koutros *et al.* linked the risk of PCa among farmers to pesticide exposure, potentially due to a mutation in chromosome 8q24.[47]

Low exposure to sunlight (ultraviolet radiation) and low levels of vitamin D may interact with prostate carcinogenesis in young men. Further investigation is crucial to understand the correlation between ultraviolet radiation exposure and vitamin D levels in combination with the risk of PCa.[43,48]

Exposure to heavy metals in the environment, such as cadmium, zinc, lead, and arsenic, may be correlated with the risk of PCa. Cadmium pollutes the environment through industrial and agricultural activities. Workers with prolonged exposure (exceeding 5 years) to cadmium have demonstrated an increased incidence of PCa compared to other populations. While mortality rates were high in several studies, this correlation was not universally observed.[49-52] Environmental exposure to zinc has not been extensively analyzed. Zinc is commonly found in water and soil. Wagner *et al.* reported that low levels of zinc concentration in the soil of certain areas of South Carolina were correlated with a high incidence of PCa in the male population.[52,53] Zinc levels were found to be 60 – 70% lower in PCa patients than in normal individuals.[43] Lead workers may also be at risk for PCa. Siddiqui *et al.* found higher lead concentrations in PCa patients.[54] Arsenic has been found in groundwaters. High levels of arsenic exposure through drinking water have been associated with an increased risk of PCa.[52]

Synthetic hormones, such as bisphenol A (BPA), can contribute to the risk of prostate carcinogenesis. BPA, a synthetic estrogen, is found in food and dental supplies. Exposure to BPA can occur through air, oral ingestion, or skin contact, potentially increasing the risk of PCa development.[10]

1.4. Link between carotenoids and cancer

The evidence suggests a protective role of carotenoids in commonly diagnosed cancers, with breast cancer comprising the most frequent type among women.[55] A meta-analysis of 33 observational studies revealed that dietary α-carotene was associated with a 9% (relative risk [RR] = 0.91, 95% confidence interval [CI] = 0.85 – 0.98) and 18% (odds ratio [OR] = 0.82, 95% CI = 0.70 – 0.97) decreased risk for breast cancer when comparing the highest with the lowest intakes, according to pooled data from cohort and case–control studies, respectively.[56] Similar results were observed for β-carotene, with the respective decrease in the risk being 6% (RR = 0.94, 95% CI = 0.88 – 1.00) and 25% (OR = 0.75, 95% CI = 0.67 – 0.85). A dose-response relationship between higher carotenoid consumption and decreased risk was revealed only for β-carotene. In addition, a pooled analysis of eight prospective studies involving 3,055 cases and 3,956 controls indicated a reduced risk of breast cancer in those in the top quantile of plasma total carotenoids (RR = 0.81, 95% CI = 0.68 – 0.96), β-carotene (RR = 0.83, 95% CI = 0.70 – 0.98), and lycopene (RR = 0.78, 95% CI = 0.62 – 0.99) compared to those in the

bottom quantile.[57] An analysis of more recent data from the Cancer Prevention Study II Nutrition Cohort also showed an inverse association between breast cancer risk and plasma α-carotene levels in postmenopausal women, which became even stronger after adjusting for multiple covariates (OR = 0.50, 95% CI = 0.29 – 0.85).[58] Finally, updated results from the Nurses' Health Study, which included 2188 breast cancer cases and 2188 controls, indicated that higher plasma concentrations of β-carotene and total carotenoids were associated with a decreased risk, both for measurements taken greater and less than 10 years before diagnosis (β-carotene: RR = 0.77, 95% CI = 0.62 – 0.96 and RR = 0.70, 95% CI = 0.51 – 0.97; total carotenoids: RR = 0.79, 95% CI = 0.64 – 0.98 and RR = 0.69, 95% CI = 0.50 – 0.95).[59]

Moreover, carotenoids have demonstrated a beneficial role in reducing the risk of lung cancer, a disease associated with the highest mortality rates.[55] A systematic review and meta-analysis comprising 24 cohort studies investigated the association between lung cancer risk and both dietary intake and serum concentrations of total and individual carotenoids.[60] Results regarding dietary intake revealed an inverse association for total and specific carotenoids, except for lutein. Specifically, when comparing the highest with the lowest intake, the pooled RR for total carotenoids was 0.79 (95% CI = 0.71 – 0.87). Similarly, the RRs for β-carotene, β-cryptoxanthin, lycopene, and lutein-zeaxanthin were 0.89 (0.79 – 1.00), 0.80 (0.72 – 0.89), 0.86 (0.77 – 0.97), and 0.89 (0.79 – 1.00), respectively. Furthermore, the dose-response analysis revealed a 1% and 2% decreased risk of lung cancer for every 0.5 and 1 mg increase in daily consumption of β-carotene and total carotenoids, respectively. However, for studies involving serum carotenoid concentration measurements and adjusting for smoking status, a statistically significant inverse association with lung cancer risk was observed only for lycopene (RR = 0.71, 95% CI = 0.51 – 0.98). In an updated systematic review with a meta-analysis of prospective studies by the World Cancer Research Fund and American Institute for Cancer Research published in 2016, 17 studies were included, comprising 3,603 cases and 458,434 participants.[60] The results indicated that increased blood levels of total carotenoids, β-carotene, and lycopene were associated with a reduced risk of lung cancer by 36% (RR = 0.64, 95% CI = 0.44 – 0.93), 29% (RR = 0.71, 95% CI = 0.56 – 0.91), and 32% (RR = 0.68, 95% CI = 0.54 – 0.87), respectively. The results of the dose-response analysis confirmed the findings for the above carotenoids and in addition α-carotene. Nevertheless, it is important to note that the results were not adjusted for smoking status, which is the most important confounding factor in this type of cancer.[61]

In contrast, the existing evidence regarding the role of carotenoids in preventing colorectal cancer, the third most common cancer globally,[55] is less promising. A pooled analysis of 11 cohort studies published in 2007 found no association between the risk of colorectal cancer and the dietary intake of individual carotenoids.[62] This finding is in agreement with the results of a recent meta-analysis published in 2017, which included both cohort (n = 4) and case–control studies (n = 11).[63] However, it is important to note that the authors reported significant heterogeneity among the studies. In addition, a meta-analysis of 15 observational studies focusing on lycopene consumption revealed that although no protective effect against overall colorectal cancer risk was observed, there was an inverse association between lycopene consumption and colon cancer (RR = 0.88, 95% CI = 0.81 – 0.96).[64] However, a key limitation across these studies is that the assessment of dietary intake of carotenoids relied primarily on food frequency questionnaires, which are susceptible to recall bias and do not account for variability in bioavailability.[63,64]

Head-and-neck cancer ranks sixth among the most common cancers,[55] and carotenoids have been suggested to have a protective effect against its development. A meta-analysis of one cohort and 15 case–control studies (5,482 cases and 14,130 controls), published in 2015, investigated the association between carotenoid intake and head-and-neck cancer at several sites.[65] The analysis revealed an inverse association between cancer of the oral cavity and pharynx and the intake of α-carotene (OR = 0.57, 95% CI = 0.41 – 0.79), β-cryptoxanthin (OR = 0.46, 95% CI = 0.29 – 0.74), and lycopene (OR = 0.74, 95% CI = 0.56 – 0.98). Similarly, a protective role of β-carotene equivalents (OR = 0.43, 95% CI = 0.24 –0.77), β-cryptoxanthin (OR = 0.41, 95% CI = 0.33 – 0.51), and lycopene (OR = 0.50, 95% CI = 0.28 – 0.89) was observed for laryngeal cancer. Furthermore, a pooled analysis conducted by the International Head and Neck Cancer Epidemiology Consortium, which included 10 case–control studies involving a total of 5959 cases and 12,248 controls, corroborated these findings.[66] Individuals with the highest intake of total carotenoids exhibited a 39% reduced risk of developing either oral and pharyngeal cancer (OR = 0.61, 95% CI = 0.53 – 0.71) or laryngeal cancer (OR = 0.61, 95% CI = 0.50 – 0.76) compared to those with the lowest intake. Moreover, increased consumption of β-carotene equivalents (OR = 0.52, 95% CI = 0.40 – 0.67 and OR = 0.55, 95% CI = 0.43 – 0.71), β-cryptoxanthin (OR = 0.62, 95% CI = 0.52 – 0.74 and OR = 0.73, 95% CI = 0.59 – 0.89), and combined lutein and zeaxanthin (OR = 0.79, 95% CI = 0.67 – 0.93 and OR = 0.73, 95% CI = 0.59 – 0.90) was associated with a reduced risk of oral, pharygeal, and laryngeal cancers.

1.5. Link between carotenoids and PCa

Numerous epidemiological studies have indicated that increased carotenoid serum levels are linked to a reduced risk of PCa incidence and progression. In a prospective study involving 450 PCa patients and 450 healthy individuals, the relationship between plasma carotenoids and PCa occurrence was investigated using blood samples and dietary habit questionnaires. The results indicated that, except for elderly participants and those without a cancer-related family history, elevated plasma lycopene concentrations were significantly associated with a lower risk of PCa. In addition, the findings imply that diets high in β-carotene might help prevent the development of PCa in younger men.[67] In another cas–control study, blood serum samples from 118 non-hispanic Caucasian males and 52 controls were analyzed using high-performance liquid chromatography to assess plasma carotenoid levels and the risk of PCa. The study found that high concentrations of cis-lycopene isomers were adversely associated with PCa risk, whereas high plasma levels of alpha-carotene, beta-carotene, alpha-cryptoxanthin, lutein, and zeaxanthin were correlated with a 50% reduced risk compared to men with lower levels.[68] Similar findings were reported in a population-based case–control study involving 193 PCa patients and 197 healthy men, which showed that elevated serum levels of lycopene, lutein, and beta-cryptoxanthin were associated with a reduced PCa risk.[69] Umesawa *et al.* investigated the link between vegetable and carotene consumption and PCa risk in a Japanese prospective study involving 15,471 male participants. A questionnaire that included questions on food consumption was administered, leading to the identification of 143 cases of incident prostate malignancies. While vegetables were not associated with a risk for PCa, moderate-to-high consumption of α-carotene may be related to a decreased risk.[70] In another Dutch cohort study involving 58,279 participants, the link between carotenoids, retinol, vitamins E and C, and PCa risk was examined. A total of 642 incident PCa cases were included in the study. Only β-cryptoxanthin had a beneficial association with a lowered risk of disease, whereas the consumption of vitamins E, C, and retinol had no impact.[71]

2. Overview of carotenoids

2.1. Substance

Carotenoids are natural isoprenoid compounds commonly found in flowers, leaves, and seeds in nature, as well as in fruits and vegetables within the human diet. Due to their characteristic color, carotenoids are often referred to as pigments. For instance, β-carotene, a precursor to vitamin A, imparts the orange color to carrots.[72] The most commonly studied carotenoids include α-carotene, β-carotene, lycopene, lutein, and β-cryptoxanthin. Specifically, α-carotene, β-carotene, and lycopene belong to the class of carotenes and are fat soluble, while lutein and β-cryptoxanthin belong to the class xanthophylls and are relatively water soluble due to the presence of hydroxyl or keto groups at the end rings.[73] In general, carotenoids are considered lipophilic and non-polar due to their large hydrocarbon structure. Figure 1B and C illustrate the main skeleton structure, which can be modified through cyclization, hydronation, or the addition of one or more oxygens. In fact, carotenoids that contain one or more oxygens are known as xanthophylls. Carotenoids can be further classified as cis or trans isomers based on the different configurations around the C-C bond. The presence of isomers can be indicated by their different melting points, solubility, and ultraviolet properties. Additional properties include different molecular geometry, thermostability, and absorption properties.[74-76] Since carotenoids are often found in ester or diester form, saponification is required after the extraction of pigments. To date, more than 600 carotenoids have been identified, but only approximately 40 of them are present in the human diet.[77] Figure 1 demonstrates the chemical structure of α-carotene, β-carotene, lycopene, lutein, and β-cryptoxanthin.

2.2. Consumption

Carotenoid consumption is generally well tolerated by the majority of the global population. According to García-Closas *et al.*, root vegetables and green leafy vegetables, such as spinach, chard, and lettuce, are the main sources of α-carotene, β-carotene, and lutein, while tomatoes and citrus fruits contribute to the majority of lycopene and β-cryptoxanthin intake.[78] However, the main source of carotenoid intake may differ from country to country, depending on their fruit and vegetable consumption patterns. A cross-sectional Spanish cohort study revealed that carotenoid consumption in the United States is one-fifth of that in Spain, reflecting the difference in carotenoid intake.[79] For instance, an average adult in the United States consumes 4.5 mg of lycopene and 1.5 mg of lutein daily. As a point of reference, one serving of tomato sauce provides 6.9 mg of lycopene, while one egg yolk provides approximately 1 mg of lutein. Safety levels for carotenoid intake are established at 75 mg of lycopene and 20 mg of lutein per day.[80]

It is important to note that the biological activity of carotenoids is highly dependent on their absorption mechanisms. Owing to their lipophilic and non-polar nature, the absorption of carotenoids requires the presence of dietary fat. This process is facilitated by their incorporation into lipid droplets, which are then absorbed by enterocytes. Such absorption is aided by bile salts and calcium, which promote micellization and reduce the size

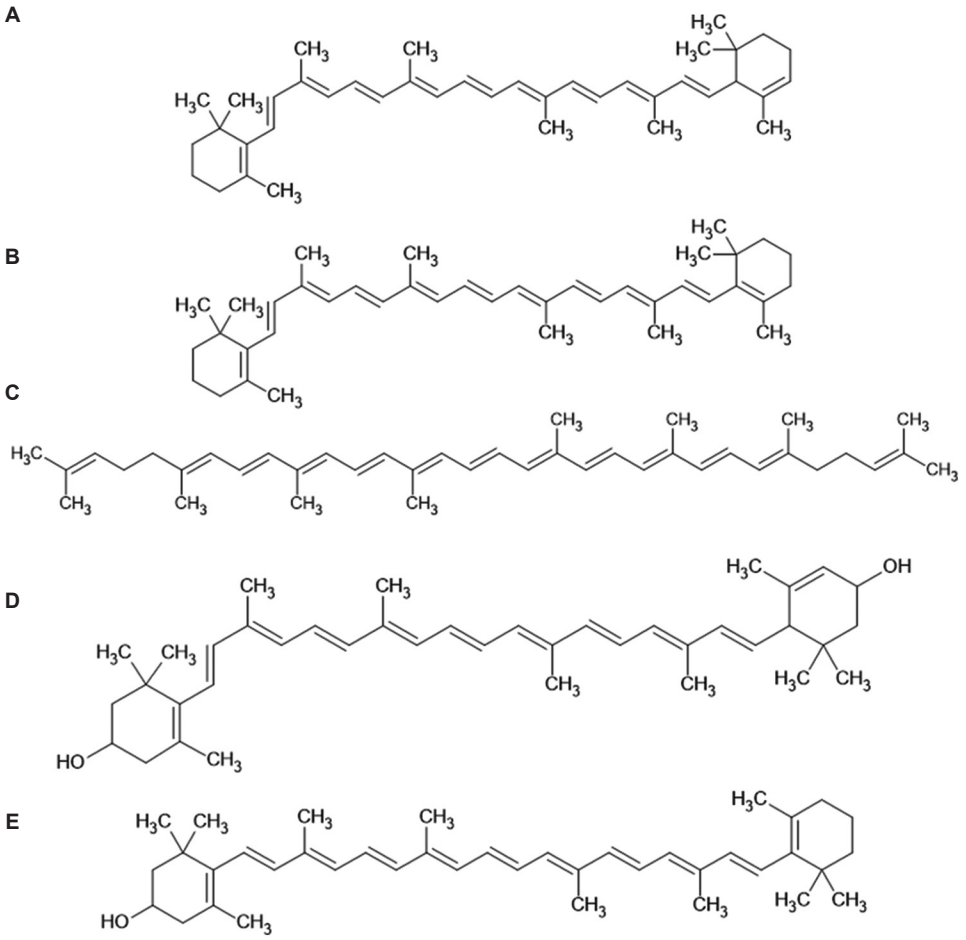

Figure 1. Chemical structure of the most commonly studied carotenoids. (A) α-carotene, (B) β-carotene, (C) lycopene, (D) lutein, and (E) β-cryptoxanthin.

of lipid droplets.[81] In addition, receptor-mediated transport serves as another mechanism facilitating carotenoid digestion and absorption, whereby carotenoids are stored in triacylglycerol-rich chylomicrons and transported to the liver.[82] Multiple factors can influence the absorption of carotenoids, with the food matrix being among the most prominent. Carotenoids display varying absorption profiles depending on the type of food or even within the same food type.[83] Notably, pectin, a component of fiber, has been identified as a factor that reduces the bioavailability of carotenoids. According to Riedl *et al.*, citrus pectin reduced the bioavailability of β-carotene by 42% and exerted a more pronounced effect compared to insoluble fiber.[84] Subsequent studies have consistently demonstrated the negative effect of pectin on carotenoid absorption.[85,86]

Moreover, both extrinsic and intrinsic factors appear to affect plasma levels of carotenoids. Extrinsic factors include diet-related elements, such as food processing, interactions with prescription drugs, alcohol consumption, and smoking, while intrinsic factors include age, hormone levels, and single nucleotide polymorphisms (SNPs).[87] Observational studies indicate that health status, such as viral infections, thyroid disorders, and respiratory conditions, also plays a significant role in determining carotenoid plasma levels. Indeed, patients with asthma or hypothyroidism often exhibit elevated levels of carotenoids.[88,89] Conversely, patients with a history of obesity, malaria, and human immunodeficiency virus (HIV) infection[90-95] tend to display lower carotenoid levels. Genetic background also plays a significant role in carotenoid absorption and metabolism. *In vivo* data suggest that women bearing double 267S and 379V mutations of the 15,15'-monoxygenase 1 (*BCMO1*) gene exhibit a poor converter phenotype, with a 69% lower ability to convert β-carotene to vitamin A.[96] Several other studies have investigated the role of combination SNPs in the bioavailability of β-carotene, lycopene, and lutein.[97-99]

2.3. Potential mechanisms of carotenoids on PCa

The potential mechanisms of carotenoids have been elucidated through multiple *in vitro* studies. However, it

is necessary to highlight that carotenoids are susceptible to oxidation and degradation induced by heat or light, a phenomenon known as photooxidation.[100] Consequently, experimental evidence should be interpreted with caution.

Laboratory studies conducted on mouse models and cell lines have revealed that β-carotene and lycopene possess apoptotic effects on PCa cells, accompanied by reduced cellular accumulation and adhesion. Specifically, the administration of lycopene at concentrations exceeding 1.25 μM resulted in reduced PCa cell growth by inhibiting the expression of NF-κB, TP53, BAX, and BCL-2 transcripts. Conversely, concentrations below 0.5 μM demonstrated no effect, thus explaining the discrepancies observed in previous studies.[101,102] Immunofluorescence staining results further demonstrated that lycopene inhibits TNFα-induced NF-κB p65 nuclear translocation in the PC3 and MDA-MB-231 cell lines, which serve as models for androgen-dependent PCa and hormone-independent breast cancer, respectively. Notably, the reduction in NF-κB activity was most prominent in the PC3 cell line compared to PCa xenografts.[103] In addition to the NF-κB p65 factor, androgen-dependent PCa appears to be regulated by the peroxisome proliferator-activated receptor gamma (PPARγ) and liver X receptor alpha (LXRα). Lycopene administration (2.5 – 10 μM) has been demonstrated to upregulate these receptors, thereby inhibiting the proliferation of PCa cells.[104] Furthermore, incubation studies demonstrated that 1.15 μmol/l of lycopene reduced PCa cell mobility by 40% and inhibited cell adhesion in concentrations exceeding 1.15 μmol/l.[105]

The majority of intervention studies investigate the synergistic chemopreventive effects of carotenoids, as administering a single compound has proven to be unsuccessful. In fact, studies by The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group and Omenn *et al.* demonstrated that supplemental β-carotene had a negative effect on smokers with lung cancer and colorectal adenoma.[106,107] However, when administered in combination with polyphenols, vitamin E, and vitamin A, β-carotene exhibited synergistic effects by reducing oxidative stress and the formation of lipid hydroperoxides and malondialdehyde.[108] Vitamin A enhances the antioxidant activity of β-carotene by eliminating oxygen radicals. Due to their lipophilic nature, carotenoids play a key role in protecting cellular membranes by eliminating peroxyl radicals generated during lipid peroxidation. Another study focusing on patients with benign breast disease followed by premenstrual mastalgia demonstrated positive results with no side effects from high-dosage supplementation of β-carotene and retinol.[109] Similarly, a combination of lycopene with phytoene, phytofluene, 1,25-dihydroxyvitamin D3, and retinoic acid at low concentrations inhibited the growth of LNCaP PCa cells. Although lycopene, phytoene, and phytofluene are effective only at high concentrations, their co-administration at low concentrations of approximately 0.3 μM demonstrated a synergistic effect.[110]

Interestingly, lycopene displays a variable anti-proliferative profile depending on the source of consumption. Lycopene derived from tomato paste and tomato extract has been shown to reduce the proliferation of PCa cells in the G0/G1 and G2/M phases. Conversely, lycopene derived from tomato sauce and ketchup leads to decreased proliferation of PCa cells in the G0/G1 phase and increased proliferation in the S and G2/M phases.[111] Overall, these results suggest the potential benefits of incorporating lycopene into the diets of prostate and breast cancer patients.[102] It is important to note that these results are sensitive to the TME and tissue specificity. Clinton *et al.* demonstrated that carotenoids exhibit variable distribution due to different transport mechanisms. For instance, lycopene tends to accumulate in the prostate, whereas zeaxanthin is mainly distributed to adipose tissue and the ovaries. Evidence indicates that carotenoids have a propensity to accumulate in tissues with low-density lipoprotein (LDL) receptors, such as adrenals, testes, and liver.[112] However, intracellular metabolic pathways also play a key role in the accumulation of carotenoids. Further pre-clinical and clinical studies are necessary to fully interpret the profile and effects of carotenoids on Pca, both *in vitro* and *in vivo*.

3. Potential use of carotenoids in patients diagnosed with PCa

Several trials have demonstrated the beneficial effects of lycopene supplementation in patients diagnosed with PCa. Randomized phase II clinical trials have indicated a reduced PCa incidence following nutritional intervention with 15 mg of lycopene administered twice a day to PCa patients before radical prostatectomy for a duration of 3 weeks. Prostate-specific antigen (PSA) levels, clinical risk assessments, and follow-up increased by 14% in the control group, whereas they declined by 18% in the group that received lycopene. In addition to reductions in PSA levels and clinical risk assessments, the intervention group also exhibited a lower prevalence of tumors with a volume of <4 mL compared to the control group. Specifically, tumors of this size were present in 84% of the intervention group and 45% of the control group.[113] In another trial, 54 patients with histologically proven metastatic PCa were enrolled. The two treatment groups respectively treated with orchidectomy alone and orchidectomy plus lycopene, each involving 27 patients. On the day of surgery, administration of 2 mg of lycopene started twice a day. PSA

levels and bone scans were obtained from patients before and every 3 months post-intervention, and the clinical response was determined by changes in these parameters. Both treatments significantly reduced PSA levels after 6 months; however, the drop in PSA levels in the lycopene group was more noticeable. These changes were observed to be more prominent 2 years after the intervention. In the orchidectomy group, only 15% of the patients exhibited a full response, compared to 30% in the other groups, according to bone scans. Therefore, combining surgical resection with lycopene leads to a more consistent and maintained reduction in serum PSA levels.[114]

In an additional clinical intervention, 26 patients newly diagnosed with PCa were randomly assigned to an intervention group and a control group. The intervention group received an extract of tomato oleoresin with 30 mg lycopene, while the control group received no lycopene supplementation for 3 weeks before radical prostatectomy. After the intervention, PSA levels in the lycopene group were reduced by 18%, compared to a 14% increase in the control group. Notably, 80% of participants in the intervention group developed tumors that were 4 cc or less, whereas this percentage was 45% in the control group. High-grade prostatic intraepithelial neoplasia was detected in 67% of the patients in the intervention group compared to 100% in the control group.[115] Another study conducted between 2001 and 2002 assessed the effect of lycopene in patients with metastatic hormone-refractory PCa (HRPC). Twenty HRPC patients participated in the trial, receiving 10 mg of lycopene daily for 3 months. Among them, one patient demonstrated a full response, six patients demonstrated partial response, 10 patients remained stable, and three patients experienced disease progression. In addition, half of the patients experienced bone pain. Five patients had no pain to mild pain, while five other patients had mild to severe pain. Bone pain remained stable in five patients and worsened in one patient. Of the 10 patients, 60% were able to reduce their daily analgesic dose. Eighteen patients experienced lower urinary tract symptoms (LUTS), of whom 60% reported symptom improvement. Therefore, lycopene treatment appears to be efficient and reliable in the management of HRPC, as it controls PSA levels and reduces bone pain and LUTS.[116]

Moreover, a phase II clinical trial conducted in 2007 evaluated the effect of lycopene on PSA levels in PCa patients, either alone or in combination with soybean-derived isoflavones. Out of 71 participants, 33 received tomato extract capsules in combination with a 40 mg soy-based isoflavone capsule blend twice a day for up to 6 months, while 38 participants received the tomato extract capsules separately (15 mg of lycopene). Serum PSA levels stabilized in 95% of patients who received the lycopene regimen and in 67% of patients who received the lycopene and soy isoflavone regimen.[117] Moreover, in a randomized control trial, 79 men diagnosed with PCa were randomly assigned to receive the following for a period of 3 weeks: 30 mg/day of lycopene-containing tomato products, tomato products along with green/black tea, soy isoflavones, selenium, omega-3 fatty acids, and grape/pomegranate juice, or a control diet. According to tumor classification, the median PSA levels in intermediate-risk patients dropped considerably in the intervention group compared to controls. In addition, they found that participants with the highest rise in serum lycopene and selenium concentrations had a median PSA value reduction of 1%, whereas participants with the lowest increase in serum lycopene and selenium concentrations experienced a median PSA value increase of 8.5%. Moreover, PSA levels were lower in patients who experienced the greatest increase in lycopene levels alone. These findings imply that the outcome may be influenced by the aggressiveness of the disease and the concentration of lycopene, selenium, and omega-3 fatty acids in serum. In addition, daily consumption of tomato products containing 30mg of lycopene for 3 weeks potentially reduces PSA levels.[118]

4. Challenges pertaining to the use of carotenoids

A major concern regarding the use of carotenoids is their bioavailability, which depends on their release and absorption from the food matrix. Specifically, bioavailability refers to the fraction of a compound that is released from the food matrix into the blood circulation. This process includes absorption, metabolism, transportation in the form of micelles to the gastrointestinal tract (a process known as micellization), and tissue distribution.[119] Bioavailability is dependent on multiple factors and can be estimated only after the administration of the total carotenoid dose. Lipophilicity and the presence of fiber play a key role in the micellization and digestion process, respectively. For instance, evidence indicates that xanthophylls, such as lutein, zeaxanthin, and β-cryptoxanthin, have a better absorption profile than carotenes due to the presence of oxygen, which is an electron-withdrawing group.[77] Out of the heterogeneous mixture of carotenoids, lycopene displays the strongest antioxidant activity, while lutein is the least effective.[120] The presence of oxygen is also prevalent during digestion, as stomach fluid provides a conducive environment for further lipid peroxidation, which induces carotenoid oxygen saturation.[108] Burton and Ingold have found that carotenoid activity is dependent upon the partial pressure of oxygen (pO_2), with higher pO_2 levels being associated with prooxidant activity.[121]

Carotenoid activity may also vary based on dietary habits, alcohol consumption, smoking, age, as well as lifestyle, menopausal, and social status. On the other hand, β-carotene consumption in combination with tobacco smoke has been found to exacerbate DNA oxidative damage through inflammatory cytokine production.[122,123] Therefore, the administration of heterogeneous carotenoid mixture and interindividual variations play a key role in assessing the potential of carotenoids as chemopreventive agents.

Regarding potential hepatotoxicity, carotenoids do not accumulate in the liver but instead integrate into very-LDL particles, which are subsequently converted to LDL. Carotenoids tend to accumulate in adipose tissue without any reported adverse events.[124,125] However, it is important to note that carotenoids are susceptible to degradation, which is triggered by UV radiation, heat, pO_2, tobacco smoke, etc. This degradation leads to the production of carotenoid breakdown products, which include highly reactive aldehydes and epoxides. These compounds are produced under conditions of high oxidative stress and are accountable for mitochondrial toxicity. To address this issue, additional antioxidants such as α-tocopherol, ascorbic acid, and N-acetyl-cysteine can be beneficial. These antioxidants create mild oxidative stress conditions, thereby inhibiting the prooxidant effects and enhancing the antioxidant properties of carotenoids.[126]

5. Conclusion

It is evident that the chemopreventive effect of carotenoids depends on a wide range of factors beyond their chemical structure. This review of the current literature suggests that further human-centered studies are necessary to fully uncover the potential of carotenoids. These studies should aim to determine their effective dose and assess their clinical value. In addition, research should not only focus on specific compounds but also investigate their synergistic effects and metabolites. Despite their potential mitochondrial toxicity, there have been no reports of severe carotenoid-related toxicity to date, with the exception of high oxidative stress conditions. Nevertheless, the prooxidant role of carotenoids and their associated adverse events remains to be investigated.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

PERSPECTIVE ARTICLE

Mitigating neglected zoonotic infections: A One Health approach on avian influenza in humans and animals

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Abstract

Avian influenza viruses pose a great challenge to both animal and human health. This viral disease, mainly affecting chickens and birds, poses a substantial zoonotic threat, particularly with the highly pathogenic avian influenza strain. The avian population is a key vector for viral transmission and fosters genetic changes and reassortment events that amplify the infectivity besides broadening the spectrum of host species. Infected animals shed viral particles into the environment, contributing to the widespread dissemination of the viral disease and perpetuating the persistence of viral strains. Given these factors, it is imperative to strengthen monitoring and prevention measures to curb the spread of the virus. Implementing vaccination and testing programs within the animal population, along with stringent biosecurity measures in agricultural environments, including adequate hygiene practices, controlled access to farms, and the separation of different animal species, could effectively mitigate the prevalence of circulating viruses. The measures not only reduce the risk of environmental spread but also mitigate the risk of viral transmission to humans through the One Health approach.

Keywords: Avian influenza; One Health approach; Zoonosis; Animal infectious disease; Highly pathogenic avian influenza; Low pathogenic avian influenza; Genetic drift; Genetic shift

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1. Introduction

Avian influenza, a highly contagious viral disease predominantly afflicting poultry and aquatic wild birds, stands as one of the most significant public health challenges globally due to its rapid spread and high mortality rate among infected animals.[1] From 2003 to 2023, the World Health Organization identified over 800 cases of avian influenza infection in humans, resulting in 400 deaths in more than 20 countries.[2] Nowadays, the world experienced three pandemics, including the swine flu pandemic of the 21st century, characterized by its massive global spread and rapid diffusion, reaching peak incidence within a year of its onset.[3]

The etiologic agent of this zoonosis is primarily distinguished into two major categories depending on its pathogenicity: (i) the low pathogenic avian influenza (LPAI) virus exhibits a low mortality rate and reduced infectivity; and (ii) the highly pathogenic avian influenza (HPAI) virus is capable of breaching respiratory and intestinal barriers,

spreading to all tissues through the bloodstream, and leading to a high mortality rate. At present, avian influenza poses a significant global public health concern due to its zoonotic potential. Among the various viral strains, the hemagglutinin 5 neuraminidase 1 (H5N1) subtype demonstrates a broad host spectrum, extending to humans and other mammals, where it elicits severe diseases or death.[4]

Given the above, it is imperative that new cases of avian influenza be promptly reported to health authorities, regardless of whether they involve HPAI or LPAI, due to their capacity to mutate.[5] While the precise mechanisms driving the mutation from LPAI to HPAI remain incompletely understood,[6] genetic drift and genetic shift are widely recognized as the primary evolutionary mechanisms.[7] Literature reports vary regarding the mutation rate, with some cases indicating rapid mutation,[8] while in others, LPAI viruses circulate for several months before undergoing mutations.[9] Clearly, the longer the circulation of LPAI, the greater the probability of mutation.[10] The primary transmission route for avian influenza viruses involves direct contact between animals, although transmission can also occur through intermediate hosts such as pigs and, less frequently, domestic animals, including dogs and cats. These intermediary hosts facilitate viral transmission to the human sphere (Figure 1).

Infected animals have the potential to release viral particles through feces, saliva, or nasal secretions into the surrounding environment, thereby easily contaminating water, mud, and soil.[11] Within these milieus, avian influenza viruses can survive for varying durations, ranging from several days to months, depending on environmental conditions and viral concentrations, thus perpetuating the transmission circle.[12]

The purpose of this work is to underline the One Health approach to avian influenza, acknowledging its various modes of transmission. Improving awareness of the transmission strategies employed by the zoonotic virus enables faster and more efficient monitoring of the infectious disease. Moreover, understanding the molecular mechanisms employed by the etiological agent to expand its host spectrum is of pivotal importance for the timely adoption of control measures and the design of additional prevention strategies. Emphasizing the multifaceted aspects of this disease suggests the exploration of novel research avenues aimed at tackling such a burdensome disease from a clinical and economic standpoints.

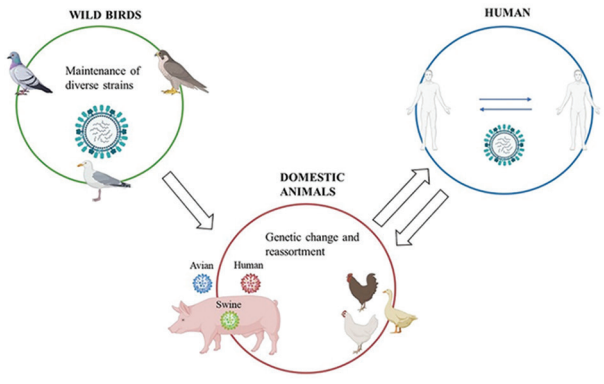

Figure 1. Transmission routes of the avian influenza virus.

2. Propagation of the avian virus in humans: Molecular basics of a potential pandemic

A major concern regarding avian influenza lies in the virus's capability to undergo genetic changes and reassortment events, which are associated with altered infectivity and adaptation to new hosts.[11] Following these changes, the virus acquires the ability to spread from human to human, thus posing a high pandemic risk.

Among the mutations known to enhance the virus's capability for human-to-human transmission, especially when shed through aerosols and/or droplets, are those affecting the glycosylation sites of the H5N1 virus.[2] These genetic modifications primarily occur in the polymerase basic (PB) 2 subunit of the polymerase, predominantly within the C-terminal domain. These genetic modifications result in amino acid exchanges, such as substitution at position 627 from glutamic acid to lysine (E627K) and substitution at position 701 from aspartic acid to asparagine (D701N). Both mutations are also responsible for the adaptation of other avian viruses to mammalian hosts, and the E627K mutation has also been identified as a determinant of airborne transmission of the H5N1 virus in ferrets.[13] Furthermore, mutations of PB 1 and PB2 subunits also have implications for drug resistance.[2] For example, Buthelezi *et al.* observed peramivir-resistant H5N1 strains with an H274Y-I222K double mutation.[14]

The increased tropism of H5N1 toward a wider range of animals implies difficulties in controlling the disease, as it consequently increases the number of virus reservoirs. These factors favor the spillover phenomenon and increase the probability of transmission to humans, potentially resulting in an outbreak or even a pandemic.[15] Finally, it could impact ecosystem dynamics by affecting various animal populations and their ecological contributions to viral transmission.[16]

3. Diagnosis of Avian Influenza: Pros and Cons

At present, the main methods commonly used for avian influenza virus detection include hemagglutination assay (HA), hemagglutination inhibition assay, reverse

transcription-polymerase chain reaction, enzyme-linked immunosorbent assay, and agar gel immune-diffusion.[2]

Among these methods, HA is considered less specific, as not all viral subtypes stimulate the production of precipitating antibodies.[17] The isolation of avian influenza viruses requires laboratories equipped with biosafety level II or III facilities. Furthermore, isolation methods are lengthy, laborious, and resource-demanding. Molecular techniques have the potential to reveal the genomic signature of the virus, which is valuable for molecular epidemiology, and nucleic acid amplification methods are the most sensitive method for H5N1 virus detection.[2]

The technology developed based on the clustered, regularly interspaced short palindromic repeats (CRISPR)-associated (CRISPR-Cas) system has recently been used for the detection of various highly pathogenic viruses, including severe acute respiratory syndrome coronavirus 2. In addition, an a CRISPR-CaS12a system has been developed for the detection of the avian influenza virus by cloning the Cas12a protein from the *Lachnospiraceae bacterium* into *Escherichia coli*.[18]

Furthermore, among the most recent methods, there are also loop-mediated isothermal amplification (LAMP)-based assay and simple amplification-based assay (SAMBA), which encompasses viral RNA extraction, isothermal DNA polymerase-facilitated DNA amplification, and subsequent detection through a dipstick system.[19] Ramos *et al.* used a 3D photopolymer microdevice for the detection of viral hemagglutinin.[20] In this technique, the biotinylated capture antibody is immobilized through a biotin-streptavidin interaction. Specifically, the analyte is labeled and subsequently interacts with a secondary antibody, forming a defined sandwich complex at the wall level.[20] The newest approaches include strategies based on next-generation sequencing[21] and the identification of new biosensors. Various biosensors have been designed and marketed to detect the presence of avian influenza virus.[22] In this regard, new biosensors capable of bypassing current diagnostic limitations are steadily being evaluated, with the aim of improving selectivity and sensitivity, as well as enabling the simultaneous detection of multiple analytes exploitable in typing the circulating viral strains.[22] Analogously to other viral infections of One Health relevance, the implementation of *in silico* approaches is desirable to expedite the optimization of diagnostic and prophylactic tools.[23]

4. Effective Control Strategies from a One Health Perspective

Important control measures are imperative as the cases of avian influenza continue to increase in both animal and human populations. As of today, the Animal Disease Information System reports more than 5000 cases of avian influenza infections in wild and domestic bird populations across. Therefore, detailed epidemiological monitoring of susceptible species and environmental and molecular factors contributing to the spread of this infectious disease is essential. Given the variety of transmission routes and the virus's capabilities of expanding the host spectra, along with the environmental implications, avian flu can be understood as a zoonotic infection of high relevance in the One Health context.[24] As such, addressing avian influenza requires the collaborative efforts of a plurality of professionals, including physicians, veterinarians, public health officials, epidemiologists, and environmental scientists. This collective endeavor is crucial for devising holistic solutions to the complex and multifaceted challenges posed by avian influenza.[25] Specifically, by adopting a collaborative and interdisciplinary approach, these professionals can work together to enhance our understanding, prevention, and control of avian flu.

Monitoring the prevalence and spread of avian flu in bird populations enables the early detection of warning signs, facilitating the implementation of appropriate measures to prevent or contain infection transmission.

Rapid detection, reporting, and containment measures are crucial to prevent the spread of the virus and minimize the risk of tropism extension. To mitigate the risk of tropism extension, surveillance systems are implemented to monitor the presence of H5N1 in animal populations, encompassing not only birds but also mammals that may come into contact with infected birds.[26,27] In addition, promoting biosecurity measures in agricultural settings is essential. These biosecurity measures include implementing proper hygiene practices, regulating access to farms, and segregating different animal species. These measures serve to reduce the risk of transmission and prevent the expansion of H5N1 tropism.

The increased sharing of the same environment between humans and animals may lead to a plausible increase in "spillover" linked to an increase in the incidence of avian influenza.

In this regard, strengthening monitoring and prevention efforts, even with systems currently undergoing testing, when applied to the animal population, could represent an indirect way of protecting the human species, thereby underlining once again the importance of a One Health approach. Furthermore, the One Health approach emphasizes the importance of vaccination measures in preventing avian influenza outbreaks.[28]

5. Conclusion

New vaccination strategies are under development to prepare for potential future pandemic epidemics by

identifying novel viral targets. This approach involves exploiting insights gained from studying immune responses to the virus. The need for identifying new targets arises from the fact that most of the vaccines currently available target hemagglutinin. However, hemagglutinin exhibits a high mutation capacity and promotes the adhesion of the virus to the carbohydrate receptors of the host cell.[29] Therefore, there is a crucial need to develop tailor-made multivalent vaccines perfectly matched to influenza virus strains with high pandemic potential. For example, mRNA-lipid nanoparticle vaccines have demonstrated the ability to induce robust immune responses in mice, rabbits, and ferrets, while also proving safe for use in humans.[29] Acknowledging the diverse range of hosts susceptible to the etiological agent, improving vaccination efforts in animal populations could effectively reduce the prevalence of circulating viruses. Consequently, this approach could decrease the risk of environmental shedding and transmission to humans, in line with the One Health concept.

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Conflict of interest

The authors declare no conflicts of interest.

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Availability of Data

Not applicable.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

High expression of apoptosis-related LMNB2 predicts an unfavorable outcome: A potential prognostic biomarker for liposarcoma

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Abstract

Liposarcoma (LPS) is the most prevalent soft-tissue sarcoma and the second most common malignant mesenchymal sarcoma. Molecular markers have proven instrumental in guiding the diagnosis, prognosis, and treatment strategies for LPS patients. Identifying potential therapeutic targets is essential for developing effective intervention strategies for LPS. *LMNB2*, an apoptosis-related gene, exhibits associations with various tumors. Therefore, exploring the feasibility of *LMNB2* as a prognostic biomarker for LPS is crucial. After screening for differentially expressed genes (DEGs), which were analyzed by GEO2R, 14 apoptosis-related genes were obtained by overlapping DEGs from the GSE21122 and GSE159659 datasets. SPSS software was used for univariate analysis. Receiver operating characteristic curves were constructed by GraphPad software to compare the expression of *LMNB2* between LPS and normal tissues. Kaplan–Meier curves were generated to verify the correlation between *LMNB2* expression and survival time. GeneMANIA and STRING were used to construct LMNB2-related gene-gene and protein–protein interaction networks. Hiplot software facilitated function and pathway enrichment analysis to determine the potential mechanism of LMNB2-mediated LPS progression. CIBERSORT was used to evaluate the correlation between *LMNB2* expression and immune cell infiltration. The expression level of *LMNB2* was significantly higher in LPS, and the high expression of *LMNB2* was significantly related to poor prognosis in LPS patients. Further analysis indicated that LMNB2 was mainly involved in "senescence" and "apoptosis," further confirming its role in regulating the occurrence and development of LPS by modulating the cell cycle progression and apoptosis. This study demonstrates that the elevated expression of *LMNB2* is significantly associated with poor prognostic outcomes in LPS, suggesting that *LMNB2* holds high potential as a new biomarker for LPS. This study is designed to elucidate the potential mechanism of LMNB2-mediated LPS progression, with the prospect of improving therapeutic development by identifying *LMNB2* as a promising prognostic biomarker for LPS.

Keywords: LMNB2; Liposarcoma; Prognostic biomarker; Apoptosis

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1. Introduction

Liposarcoma (LPS), a malignant mesenchymal tumor arising from adipocytes, accounts for approximately 15 – 20% of soft tissue sarcomas (STS) and ranks as the second most common subtype following gastrointestinal stromal tumors.[1,2] LPS is a diagnostically challenging STS due to its diverse histological subtypes, with patient prognosis and treatment strategies depending on the histological subtype. The five major histopathological subtypes of LPS include well-differentiated LPS (WDLPS), dedifferentiated LPS (DDLPS), myxoid LPS (MLPS), pleomorphic LPS (PLPS), and myxoid pleomorphic LPS.[3,4] Given the clinicopathologic discrepancies among these histological subtypes, an in-depth exploration of their clinical manifestation, treatment sensitivity, and underlying pathogenesis is imperative to guide targeted therapy strategies aimed at improving patient survival.[2,5] At present, complete surgical resection with clear margins is the most effective treatment modality for LPS. In addition, radiotherapy reduces the risk of high-grade recurrence, while chemotherapy mitigates subtypes with metastatic potential, albeit without enhanced outcomes for drug-resistant DDLPS subtypes.[6-8] In recent years, there has been significantly increased widespread attention given to the development of new drugs for targeted therapies, including exportin 1 inhibitors and PPARγ agonists.[2,8] Targeting murine double minute 2 or/and cyclin-dependent kinases 4 has been proven to be a promising therapy strategy for LPS patients.[2,9] Given the high molecular heterogeneity inherent in LPS, the identification of additional biomarkers as potential therapeutic targets is crucial for the development of effective treatment options and the improvement of patient survival time.

Lamin B2, a member of the lamin family, is encoded by the *LMNB2* gene located on chromosome 19p13.3.[10] Lamins, categorized as V-shaped intermediate filament proteins,[11-13] predominantly constitute the nuclear lamina, serving as a fundamental structural component.[13,14] Integral to the organization and functionality of the nucleus, lamins mediate the connections between the inner nuclear skeleton and the peripheral lamina, ensuring stability and morphological maintenance while facilitating signal transduction, transcription regulation, nuclear pore anchoring, and chromatin regulation.[13,15-17] Lamin B2 is implicated in a variety of cellular processes, including cell cycle control and apoptosis.[12,14,18,19] Considered a key anti-apoptotic protein,[17] Lamin B2 also participates in chromatin epigenetic regulation, nuclear membrane breakage, and recombination events during mitosis.[20] While Lamin B2 is cleaved by caspase-6, leading to its inactivation (Figure 1), increased expression delays cell death, proving its involvement before DNA fragmentation.[21,22] The nuclear events of apoptosis involve lamin cleavage and inactivation, as well as the cleavage of structural protein Gas2, which is involved in actin microfilament reorganization and DNA fragmentation. Therefore, inhibiting lamin cleavage alone is insufficient to block apoptosis, but it can promote nuclear decomposition and accelerate apoptosis[21,22] (Figure 1).

It has been reported that the abnormal expression, gene mutation, or abnormal localization of *LMNB2* can affect the nuclear scaffold structure, leading to changes in nuclear morphology and dysfunction,[11,12,23] ultimately contributing to cancer cell migration and metastasis.[11,15,24,25] The expression level of *LMNB2* has been correlated with the prognosis of tumors, including primary colorectal cancer,[20] hepatocellular carcinoma,[16] non-small cell lung cancer,[26] triple-negative breast cancer,[27] and bladder cancer.[28] However, it remains unclear whether *LMNB2* exhibits significant expression changes in LPS tissues, and the correlation between *LMNB2* and LPS prognosis requires exploration.

In this study, the differential expression patterns and prognostic significances of *LMNB2* in LPS were analyzed to identify its potential as a biomarker for LPS patients. Our findings indicate that elevated expression of *LMNB2* is a common feature in LPS and is associated with shorter survival time for LPS patients. Thus, *LMNB2* emerges as a potential prognostic biomarker and promising therapy target for LPS.

2. Methods

2.1. Data download and collation

The flowchart illustrating the study's methodology is illustrated in Figure 2. Expression profiling and survival data of LPS patients were obtained from The Cancer Genome Atlas (TCGA [https://portal.gdc.cancer.gov/]). This dataset encompassed crucial information, including survival time, age, gender, clinical stage, and Tumor, Nodes, and Metastasis stage for 60 LPS patients. In addition, expression profiling data from the GSE159659 and GSE21122 datasets were collected from the Gene Expression Omnibus (GEO) database. These datasets comprised different subtypes of normal and tumor samples. Specifically, GSE159659 contained 15 normal samples, 15 DDLPS samples, and 15 WDLPS samples, while the GSE21122 dataset contained nine normal and 149 LPS samples. Furthermore, the GSE30929 dataset included 140 tumor samples.

2.2. Differential expression analysis of microarray data by GEO2R

GEO2R (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) is an interactive web tool used for comparing gene

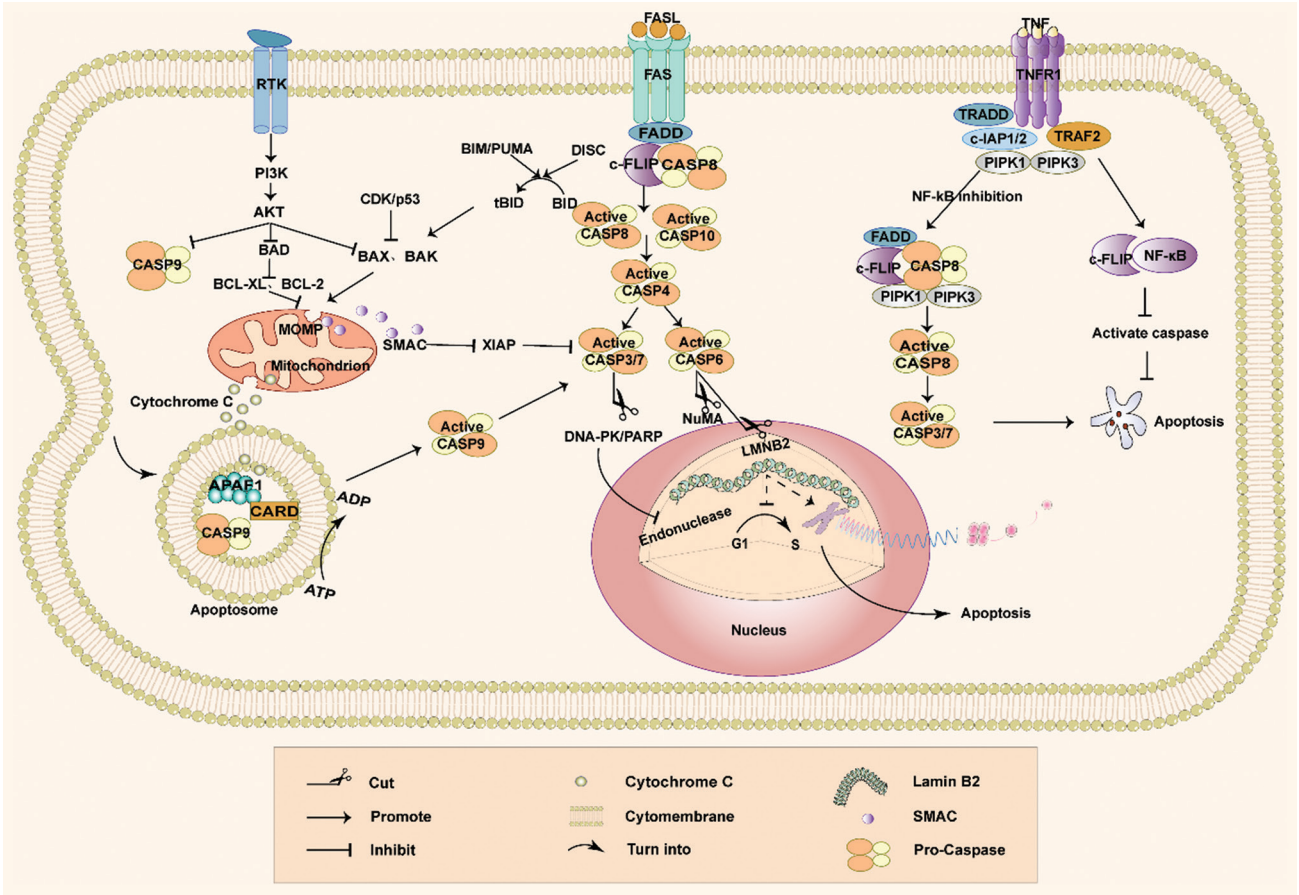

Figure 1. Schematic diagram of the regulation of apoptosis-related gene *LMNB2*. Caspase cleavage of lamin LMNB2 is one of the key nuclear events in the process of apoptosis. As an important link connecting the extrinsic and intrinsic apoptotic pathway, increased levels of BIM, PUMA, and DISC influence the cleavage of BID, forming tBID. tBID translocates to mitochondria and promotes the release of cytochrome C. BAX and BAK undergo conformational changes and aggregation on activation on the mitochondrial surface, which results in the formation of MOMP and promotes the release of apoptosis-related proteins (e.g., cytochrome C, SMAC) into the cytoplasm to activate caspase-9 and regulate the downstream effector caspase-3/7. SMAC can promote apoptosis by neutralizing XIPA, which has an inhibitory effect on the apoptotic process. The amino-terminal CARD structural domain of APAF-1 interacts to form a heptamer, which recruits, in the presence of cytochrome C, the activated caspase-9 to form apoptotic vesicles. PI3K phosphorylates AKT to inhibit BAD, caspase-9 and pro-apoptotic BCL-2 family members to promote apoptosis. Caspase-8/10 serves as a DISC initiator, which is activated by dimerization and autocatalytic cleavage, and the activation process is regulated by FLIP. The binding of extrinsic death signaling ligands to their receptors regulates the expression of apoptotic signals through two pathways. One way is to activate the target protein c-FLIP, which inhibits the activation of NF-κB, thereby inhibiting the cell survival pathway and promoting apoptosis. Another pathway recruits CASP8/10 to form DISC, which activates downstream CASP4 and CASP3/6/7, acting on different substrates (including lamin, PARP, and Rb proteins) to play different roles but ultimately promoting apoptotic progression.

Abbreviations: AKT: As known as Protein Kinase B (PKB); APAF1: Apoptotic protease-activating factor 1; BAD: BCL-2-associated agonist of cell death; BAK: BCL-2 antagonist/killer; BAX: BCL-2-associated X protein; BCL-2: B cell lymphoma 2; BCL-XL: B cell lymphoma extra large; BID: BH3-interacting domain death agonist; BIM: BCL-2-interacting mediator of cell death; CARD: Caspase recruitment domain; DISC: Death-inducing signaling plex; MOMP: Mitochondrial outer membrane permeabilization; NuMA: Nuclear mitotic apparatus; PARP: Poly ADP-ribose polymerase; PI3K: Phosphatidylinositol 3-kinase; PUMA: p53 upregulated modulator of apoptosis; SMAC: Second mitochondria-derived activator of caspases; XIAP: X-linked inhibitor of apoptosis protein.

expression differences between normal and tumor samples. We employed this tool to identify the differentially expressed genes (DEGs) within datasets GSE21122 and GES159659, respectively. Following analysis, DEGs were preliminarily screened according to the criterion: |logFC| > 1 and adj.*p*.Val< 0.05. Among them, 896 DEGs were obtained from GSE21122, while 318 DEGs were obtained from GSE259659. After overlapping these datasets, 192 co-DEGs were identified.

2.3. Gene function enrichment analysis by KEGG orthology based annotation system (KOBAS) 3.0

KOBAS [kobas.cbi.pku.edu.cn/] is a web server designed for gene function annotation and enrichment.[29-31] The

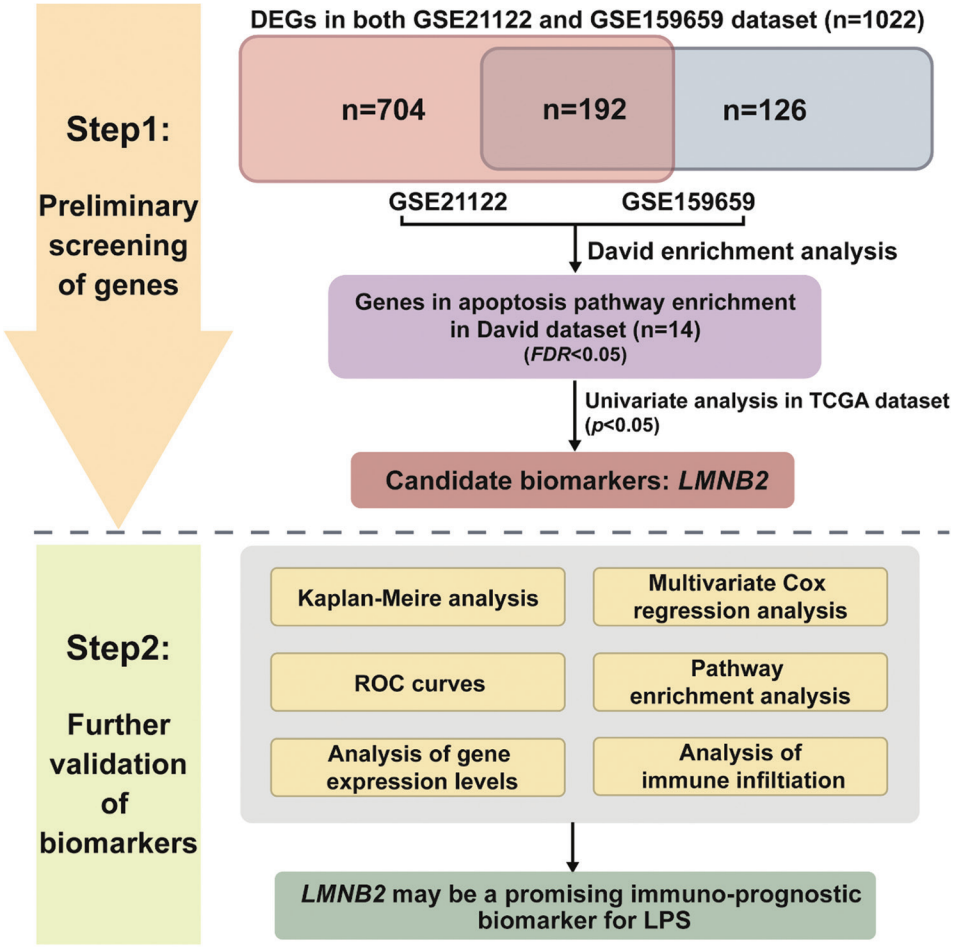

Figure 2. Research flowchart.
Abbreviations: DEGs: Differentially expressed genes; *FDR:* false discovery rate; LPS: Liposarcoma; ROC: Receiver operating characteristic; TCGA: The Cancer Genome Atlas.

annotated database includes pathways, diseases, and gene ontology (GO) terms. Enrichment analysis includes both gene-list enrichment and experimental data enrichment. In this study, we utilized the enrichment function of the KOBAS database to perform Kyoto Gene and Genome Encyclopedia (KEGG) pathway enrichment analysis for genes associated with the apoptotic pathway. First, the NCBI Entrez gene ID was selected as the gene-list type, with the corresponding species set as human. Eventually, "KEGG Pathway" and "KEGG Disease" were selected as the enrichment categories. After execution, the resulting file contained the enriched pathways related to apoptosis, encompassing a total of 14 genes ($P < 0.05$).

2.4. Co-expressed genes and protein-protein interactions (PPIs) exploration by GeneMANIA and STRING

GeneMANIA (http://genemania.org/) is a website specialized in predicting gene/protein interaction. It utilizes functional association datasets to predict gene functions and identify other genes associated with input genes.[32-34] In this study, we utilized the function enrichment and network classification capabilities of this database to analyze DEGs obtained from the preliminary screening. GeneMANIA identifies genes associated with *LMNB2* and closely related to apoptosis based on gene-gene interactions and displays the weights of the predicted values of genes with functions similar to *LMNB2*.[34]

STRING (https://string-db.org/) is a database dedicated to searching PPIs. It includes both direct physical PPI and indirect functional correlations between proteins.[35] The database contains experimental data derived from PubMed abstract, comprehensive data from other databases, and analysis results predicted by bioinformatics methods.[36] We uploaded 21 genes, including *LMNB2*, obtained from GeneMANIA to the STRING database and generated PPI network diagrams.

2.5. Analysis of immune infiltration by CIBERSORT

CIBERSORT (https://cibersortx.stanford.edu/) is a tool designed for the deconvolution of expression matrices of human immune cell subtypes. It operates on the principle of linear support vector regression, using gene expression data to estimate the abundance of cell types within a mixed cell population.[37,38] After uploading a microarray or sequencing expression matrix and a reference dataset, CIBERSORT generates outputs indicating the proportion of immune cell infiltration based on the reference dataset. In addition, it provides statistics such as *P*-values, *R*, and *RSME*, which sum to one for all cell types under default parameters.[39] In our study, CIBERSORT was employed to analyze immune infiltration in tumor tissues obtained from 60 LPS patients in the TCGA dataset. This analysis yielded insights into the percentage of immune cell infiltration based on the reference dataset. The results obtained were further visualized using GraphPad software, which provides new insights into biomarkers associated with LPS pathogenesis and prognosis.

2.6. Statistical analysis

IBM SPSS Statistics 26 (SPSS Inc., USA) and GraphPad Prism 8.4.3 (GraphPad Inc., USA) were used for statistical analysis. Univariate and multivariate Cox regression models were developed using SPSS software. In the univariate analysis, biomarkers significantly associated with LPS prognosis were identified, with risk factors screened ($P < 0.05$).[40] Subsequently, meaningful risk factors identified in the univariate analysis were also subjected to a multifactorial analysis, with a significance level of $P < 0.05$ employed as the criterion for statistical significance. Survival curves for LPS patients from the TCGA or GSE30929 dataset were plotted by GraphPad software with a 50% cutoff value.

3. Results

3.1. Preliminary screening of LPS prognostic biomarker

Through GEO2R, DEGs were obtained from GSE21122 and GSE159659 (with a significance threshold of $P < 0.05$ and |FC| >2). After overlapping, 192 DEGs were acquired by intersecting 318 DEGs in GSE159659 and 896 DEGs in GSE21122 (Figure 2). The resulting gene list was then imported into the KOBAS database for pathway enrichment analysis. A meaningful apoptosis pathway was identified in the exported result file (false discovery rate [*FDR*] <0.05), comprising 14 genes, including *LMNB2*, *LMNB1*, *FOS*, *GADD45B*, *NFKBIA*, *JUN*, *BAX*, *MAP3K5*, *PIK3R3*, *MCL1*, *PIK3R1*, *ATF4*, *ITPR1*, and *BIRC5*. Subsequently, SPSS software was used for univariate analysis of these 14 genes. Among them, only *LMNB2* exhibited a |FC| <1 and $P < 0.05$ ($P = 0.013$, HR = 3.117, 95% confidence interval [CI, 1.271 – 7.645]), while other genes did not meet this statistical criterion (Figure S1). Therefore, we initially considered *LMNB2* as a potential LPS biomarker and proceeded with further investigations.

3.2. Significantly upregulated *LMNB2* in LPS tissues

To investigate the potential prognostic value of *LMNB2* as a biomarker for LPS, we analyzed the expression of *LMNB2* in different subtypes of LPS tissues and normal tissues using GSE21122 and GES30929 datasets. The results revealed a significant increase in *LMNB2* expression in LPS tissues compared with normal controls (Figure 3A). Moreover, the expression of *LMNB2* was upregulated across different subtypes of LPS tissues compared with normal tissues, suggesting a close association between the increased expression of *LMNB2* and the development of each subtype of LPS (Figure 3B). In addition, significant differences were observed in the expression of *LMNB2* among different LPS subtypes, especially between WDLPS and other subtypes. Notably, the expression of *LMNB2* was significantly higher in DDLPS or PLPS than Myxoid/round cell LPS, indicating a potential correlation between the expression of *LMNB2* and the occurrence of different LPS subtypes (Figure 3B and C). The observed significant differences in the expression of *LMNB2* between normal and LPS tissues, as well as among different LPS subtypes, suggest a potential role for LMNB2 in the occurrence and development of LPS. In addition, analysis of *LMNB2* expression in DDLPS patients revealed significantly higher expression of *LMNB2* in patients with copy number amplification ($n = 21$) than those with normal diploid copy number ($n = 32$) ($P = 0.003$) (Figure S2), indicating a consistent relationship between DNA copy number of *LMNB2* and its expression level.

Next, receiver operating characteristic curves were generated based on the expression levels of *LMNB2* in LPS and normal tissues of each subtype. The results revealed a significant increase in LPS tissues compared with normal controls (Figure 3D). Furthermore, the expression of *LMNB2* was increased across various subtypes of LPS tissues compared with normal tissues (Figures 3E and S3A-S3B). In addition, significant differences in *LMNB2* expression were observed among different LPS histological subtypes (Figures 3F and S3C-S3F). These results indicate that the expression of *LMNB2* differs significantly between normal and LPS tissues, as well as among LPS histological subtypes. This finding suggests that the expression of *LMNB2* demonstrates a good discrimination ability for diagnosing LPS or differentiating LPS subtypes, and it might be related to the occurrence and development of LPS.

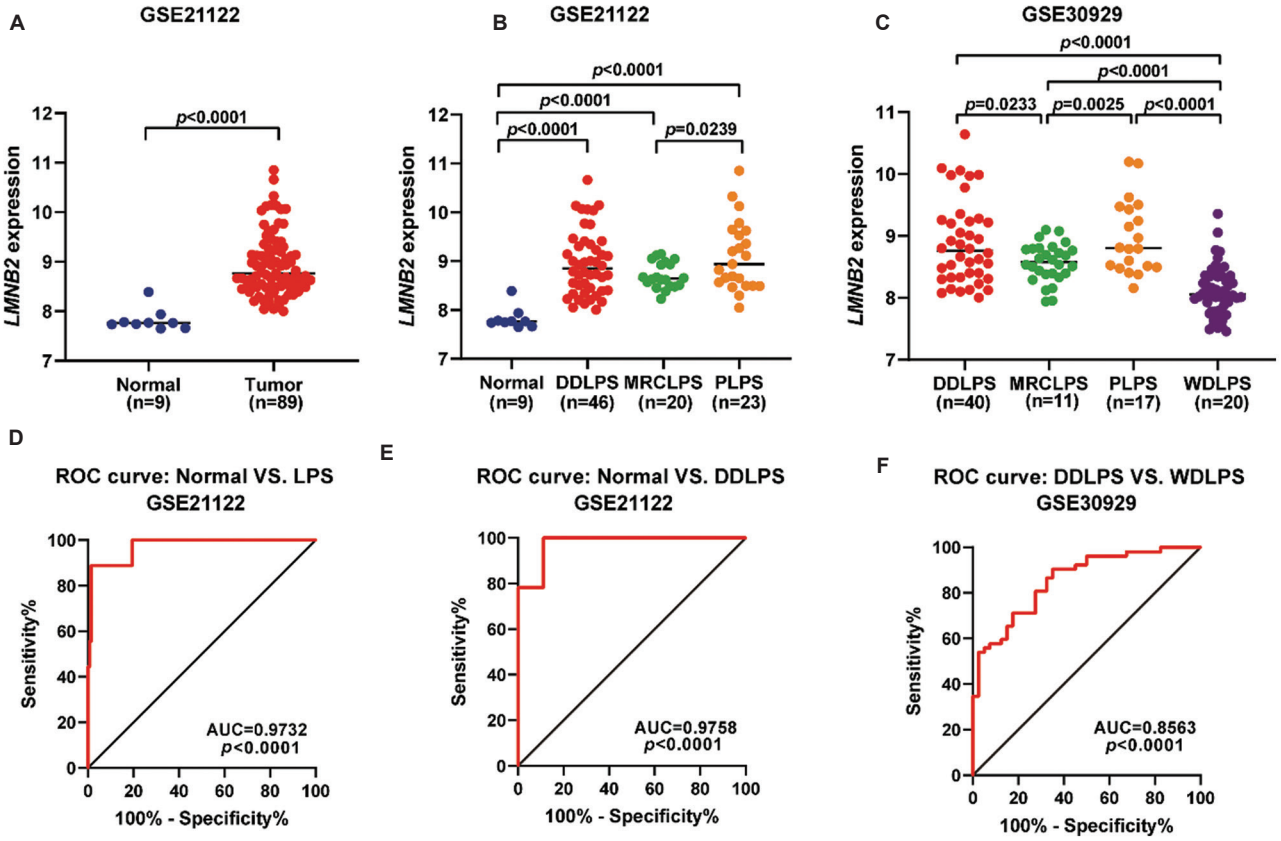

Figure 3. Expression features and ROC curves of *LMNB2* in different subtypes of LPS. (**A**) Differences in *LMNB2* expression levels between normal and tumor tissues. (**B and C**) The level of *LMNB2* expression varies in patients with different subtypes. (**D and E**) ROC curves of the expression of *LMNB2* in normal and LPS tissues. (**F**) *LMNB2* expression discrimination between DDLPS and WDLPS.
Abbreviations: DDLPS: Dedifferentiated liposarcoma; LPS: Liposarcoma; ROC: Receiver operating characteristic; WDLPS: Well-differentiated liposarcoma.

3.3. Significant correlation between *LMNB2* expression and poor prognosis in LPS patients

The prognostic value of *LMNB2* was further determined by mapping Kaplan–Meier curves for overall survival (OS) and distant relapse-free survival (DRFS) in LPS patients. In patients with DDLPS, the high expression of *LMNB2* predicted poor OS ($P = 0.0091$) (Figure 4A) and DRFS ($P = 0.0198$) (Figure 4B). In addition, patients with higher *LMNB2* expression exhibited significantly shorter DRFS time than those with lower *LMNB2* expression across all subtypes ($P = 0.0003$) (Figure 4C), consistent with the correlation observed between the two survival curves of DDLPS (OS and DRFS).

Moreover, we also plotted Kaplan–Meier curves for patients with different clinicopathological characteristics, including age ≥60 ($P = 0.0058$) (Figure 4D), male gender ($P = 0.0028$) (Figure 4E), and absence of new tumor events after initial treatment ($P = 0.0381$) (Figure 4F). The survival time significantly differed between the high and low *LMNB2* expression groups across these pathological characteristics.

To determine whether the expression of *LMNB2* could serve as an independent risk factor for LPS prognosis, both univariate and multivariate analyses were conducted. The univariate analysis revealed that *LMNB2* expression and therapy outcome (progressive disease) were significantly associated with a higher prognostic risk (Table 1). Subsequent multivariate analysis further revealed that *LMNB2* expression or therapy outcome (progressive disease) could be considered independent prognostic risk factors for LPS (Table 1).

3.4. Construction of gene-gene and PPI network of *LMNB2*

A gene-gene interaction network of *LMNB2* was constructed, and its function was analyzed using the GeneMANIA database (Figure 5A). At the mRNA level, the *LMNB2* gene is surrounded by 20 nodes that represent the top 20 genes associated with *LMNB2* in terms of physical interactions, coexpression, predictions, co-localization, and genetic interactions (Figure 5A). The

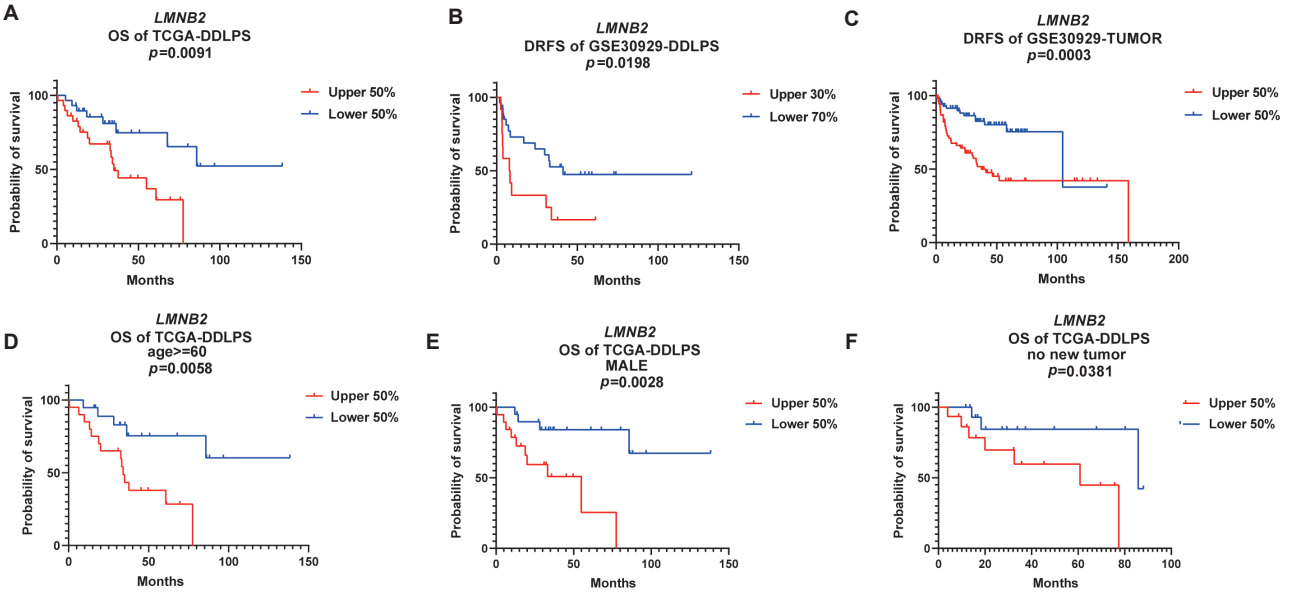

Figure 4. Kaplan–Meier (K-M) survival analyses of *LMNB2* differential expression groups. (**A**) K-M curves compare the survival of patients with high vs. low *LMNB2* expression. (**B and C**) K–M curves of DRFS time of the patients with LPS and DDLPS. (**D-F**) The K-M curves show the survival status of high and low *LMNB2* expression groups under different clinicopathological features, including age, gender, and recurrence.
Abbreviations: DDLPS: Dedifferentiated liposarcoma; DRFS: Distant relapse-free survival; LPS: Liposarcoma; OS: Overall survival; ROC: Receiver operating characteristic; WDLPS: Well-differentiated liposarcoma; TCGA: The Cancer Genome Atlas.

Table 1. Univariate and multivariate analysis of risk factors associated with liposarcoma survival

Subgroup (All patients [*n*=59])	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% confidence interval)	*P*-value	Hazard ratio (95% confidence interval)	*P*-value
LMNB2 expression: High versus low (*n*=58)	3.117 (1.003 – 5.235)	0.013*	2.840 (1.147 – 7.031)	0.024*
Therapy outcome: Progressive disease versus complete response (*n*=33)	9.046 (2.009 – 40.792)	0.004*	7.448 (1.737 – 31.946)	0.007*
Female versus male (*n*=59)	0.501 (0.203 – 1.008)	0.081	-	-
Age ≥ge versus <60 (*n*=59)	0.538 (0.202 – 1.492)	0.213	-	-

Note: **P*<0.05.

size of nodes indicates the strength of the interaction, while the connecting lines between nodes indicate the type of gene-gene interaction. In addition, the color of the lines indicates the type of interaction. Notably, the first two genes most closely related to *LMNB2* are *LMNA* and *LMNB1* (Figure 5A). At the protein level, a PPI network was mapped (Figure 5B), revealing the top ten genes closely related to *LMNB2*: *LMNA* (score = 0.975), *LMNB1* (score = 0.971), *CASP6* (score = 0.950), *LEMD3* (score = 0.930), *CASP2* (score = 0.922), *LBR* (score = 0.920), *SUN1* (score = 0.920), *BANF1* (score = 0.889), *EMD* (score = 0.872), and *TM7SF2* (score = 0.828). In the identified *LMNB2* coexpression gene and PPI networks obtained using GeneMANIA and STRING, we observed that the top two genes, *LMNA* and *LMNB1*, belong to the laminin family, similar to *LMNB2*.

Based on the constructed gene–gene interaction network, GO annotation and KEGG pathway enrichment analysis were conducted using Hiplot software. Our analysis revealed that LMNB2 exhibits significant enrichment in various biological processes (BPs), with the most notable functions including "histone modification" and "aging" (Figure 5C). In terms of molecular function, LMNB2 demonstrates a high level of enrichment in "cysteine endopeptidase activity and apoptosis process" and "protein phosphorylated amino acid binding" (Figure 5D). Regarding cell components, LMNB2 is predominantly enriched in structures such as the "nuclear envelope," "intermediate filament," and "intermediate filament cytoskeleton" (Figure 5E). Furthermore, our KEGG pathway enrichment analysis identified "apoptosis" as the primary enriched pathway for LMNB2, consistent with the abovementioned results (Figure 5F). Therefore, the main

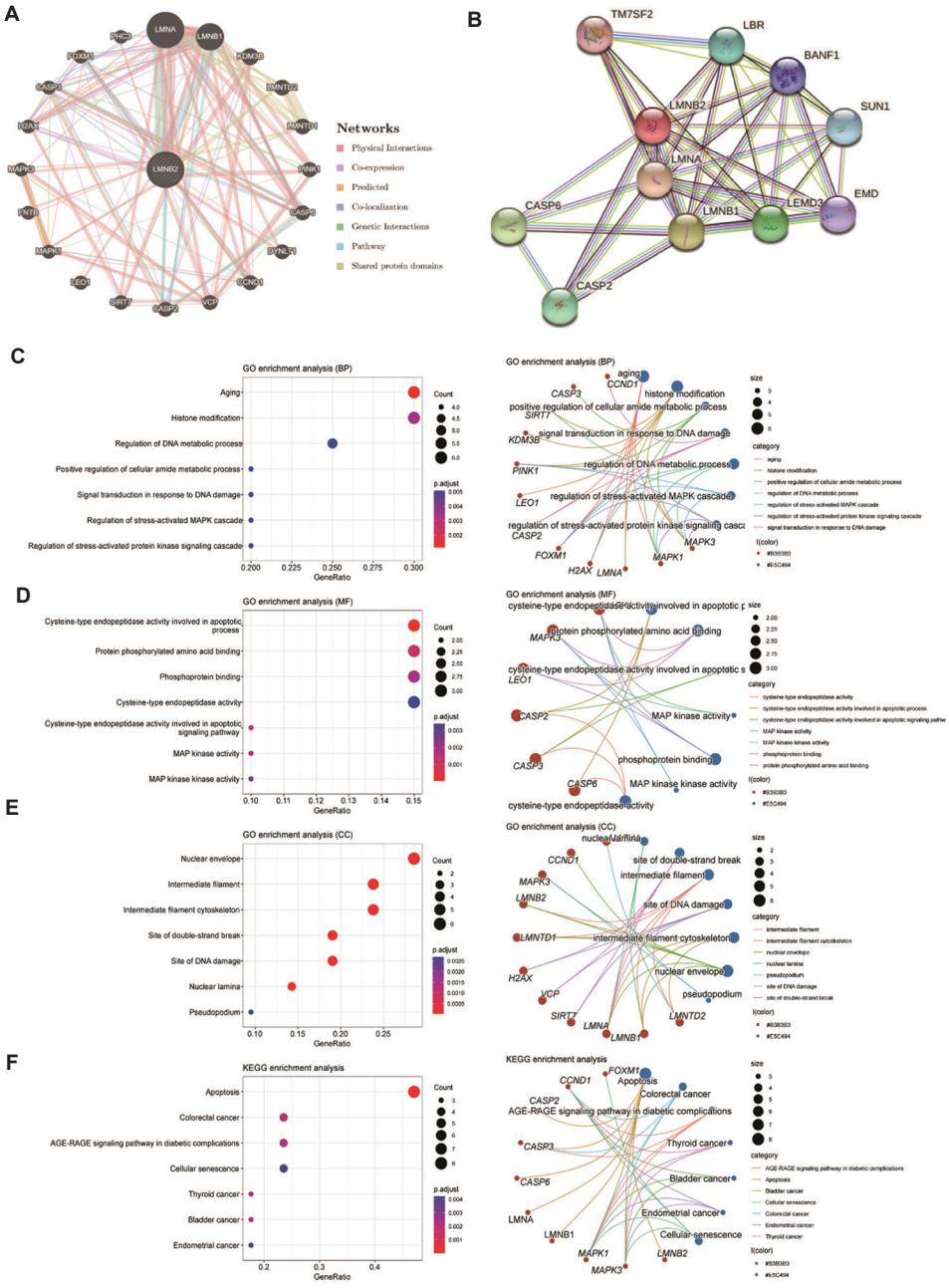

Figure 5. Gene ontology (GO) enrichment analysis of coexpressed genes of *LMNB2*. (**A**) The gene–gene interaction network among genes related to *LMNB2*. (**B**) The top 10 predicted protein-protein interactions related to *LMNB2*. (**C**-**F**) Bubble plot and gene-concept network of BP, MF, CC, and KEGG enrichment analysis, respectively.
Abbreviations: BP: Biological process; CC: Cell component; KEGG: Kyoto Gene and Genome Encyclopedia; MF: Molecular function.

function of LMNB2 in the BP is to participate in cysteine endopeptidase activity and apoptosis process.

3.5. *LMNB2* gene set enrichment analysis

To further explore the mechanism of LMNB2 in LPS progression, we used expression profile data of DDLPS patients for single-gene enrichment analysis (Figure 6A). A total of 176 gene sets were found to be enriched, with 56 gene sets showing enrichment in the group exhibiting high expression of *LMNB2* (Figure 6B). It was found that the gene sets enriched in the high expression group include "cell cycle" (Normalized enrichment score [NES] = 1.984, $P < 0.0001$) and "oocyte meiosis" (NES = 1.870, $P = 0.006$), which are closely associated with the progression of LPS

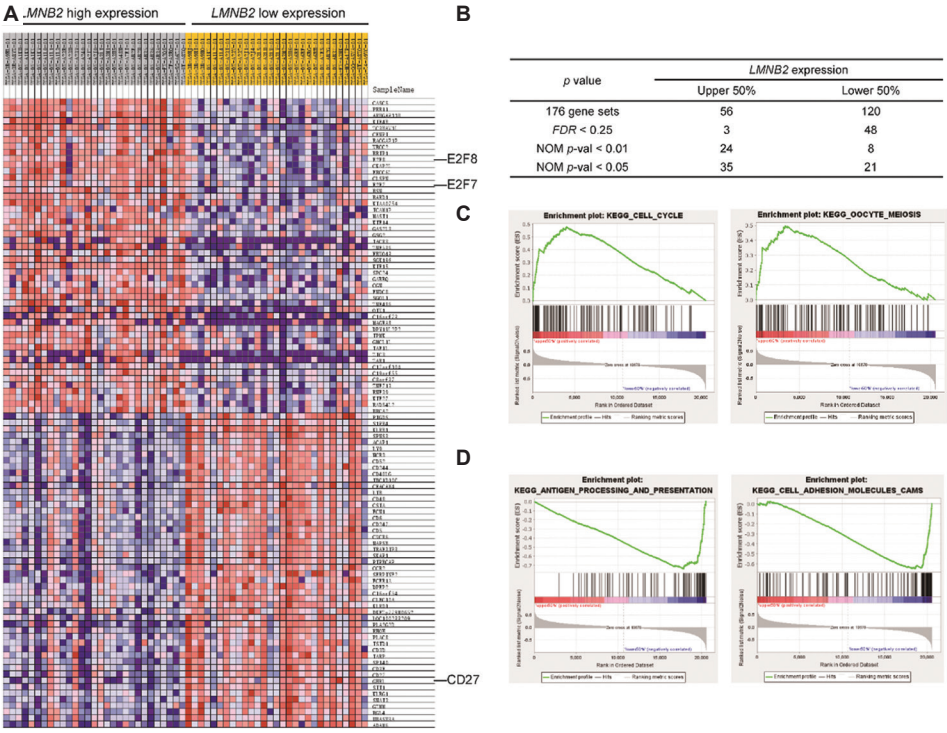

Figure 6. Gene set enrichment analysis (GSEA) analysis of *LMNB2*. (**A**) Heat map of GSEA analysis of *LMNB2*, which includes the top 100 genes enriched in high and low *LMNB2* expression. Red represents upregulated genes; Blue represents downregulated genes. (**B**) A summary of GSEA analysis of *LMNB2*. (**C**) Cell cycle and oocyte meiosis pathway. (**D**) Cell adhesion molecules and antigen processing and presentation pathway.

(Figure 6C). Conversely, the gene sets enriched in the low expression group consist of "antigen processing and presentation" (NES = −1.836, $P < 0.0001$) and "cell adhesion molecules cams" (NES = −1.848, $P = 0.004$) (Figure 6D). When *LMNB2* is highly expressed, both *E2F7* and *E2F8* also exhibit high expression (Figure 6A).

3.6. Correlation between LMNB2 expression and immune cell infiltration in LPS

Subsequently, CIBERSORT was used to assess the difference in infiltration levels of 22 types of immune cells between the high and low *LMNB2* expression groups (Figure 7A and B). Results revealed that compared with the low *LMNB2* expression group, the infiltration level of "macrophage M0" was higher in the high expression group ($P = 0.012$), while the infiltration level of "T cells CD8" was lower in the high expression group ($P = 0.026$) (Figure 7A and B). To further study the relationship between the expression level of *LMNB2* and the immune infiltration level, the TIMER2.0 database was applied to evaluate the correlation between immune cell infiltration and survival in sarcoma (including LPS). The results illustrated a significant association between the infiltration level of "B cell" (log-rank $P = 0.455$), "CD4+T cell" (log-rank $P = 0.002$), or "Neutrophil" (log-rank $P = 0.014$) and the survival of patients with sarcoma (Figure S4A). At the same time, we also found a significant correlation between the expression level of *LMNB2* and the infiltration level of "CD8+T cells" (Figure S4B). Furthermore, we plotted the Kaplan–Meier curves depicting immune cell infiltration and survival of sarcoma patients under conditions of high or low *LMNB2* expression. When *LMNB2* was highly expressed, the infiltration level of "Macrophage M2" ($P = 0.0101$), "Macrophage/Monocyte" ($P = 0.00228$), or "CD4+T cell" ($P = 0.000118$) exhibited statistical significance with respect to the survival of sarcoma patients (Figure S4C). In addition, the TISIDB database was used to study the relationship between the abundance of tumor-infiltrating lymphocytes (TILs) or immune stimulators and the expression of *LMNB2* (Figure S4D and E). The lymphocytes most closely related to *LMNB2* expression were "CD4+T cells" ($P = 3.08e-07$) and "Th2 cells" ($P = 4.26e-09$). As depicted in Figure S4E, the immune stimulators most closely related to the expression of *LMNB2* are ULBP1 ($P = 8.21e-08$), TNFRSF14 ($P = 3.18e-08$), CD40L ($P = 5.08e-11$) and ENTPD1 ($P = 3.15e-13$).

In addition, we also plotted a lollipop map illustrating the correlation between multiple immune checkpoint-related genes and the expression of *LMNB2* (Figure 7C).

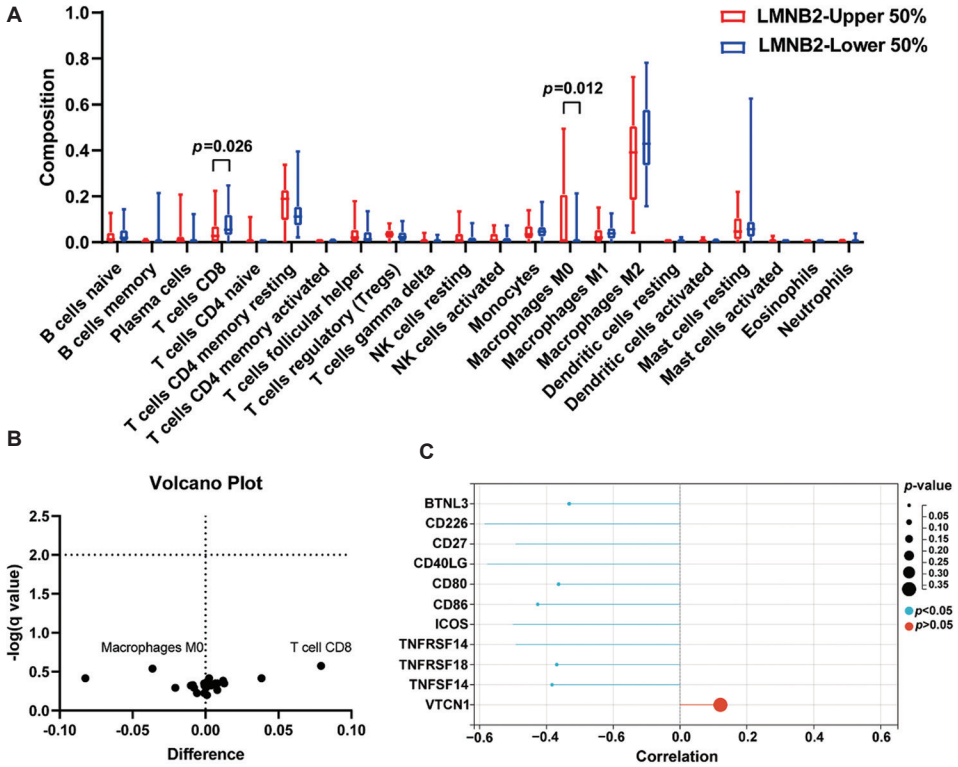

Figure 7. Immuno-infiltration analysis and correlation analysis of immunological checkpoints. (**A**) Differences in infiltration levels of 22 types of immune cells between LMNB2 high and low expression groups. (**B**) Volcano Plot of infiltration levels. (**C**) Correlation analysis between immune checkpoint gene and *LMNB2* expression.

In Figure 7, the length of the line represents the correlation between the immune checkpoint gene and *LMNB2* expression, while the size of the node represents the size of the *P*-value. Green nodes represent a *P*-value <0.05, while red nodes represent a $P > 0.05$. As shown in Figure 7C, the expression of stimulating molecules such as BTNL3,[41] CD226,[42] CD27,[43] CD40LG,[44] CD80, CD86,[45] ICOS,[46] TNFRSF14,[47] TNFRSF18, or TNFRSF18[48] is negatively correlated with the expression of *LMNB2*, showing a strong correlation and statistical significance. Conversely, the expression of the inhibitory molecule VTCN1[49] positively correlates with *LMNB2*. Therefore, we speculate that the poor prognosis of patients with high *LMNB2* expression may be related to lower expression of stimulatory molecules and higher expression of inhibitory molecules.

4. Discussion

Due to the heterogeneity of LPS, the recurrence rate exceeds 80%,[50] while the mortality rate varies with the prognosis depending on the histological subtype.[51] As the second most common subtype of LPS, MLPS accounts for about 15 – 20% of LPS cases with higher recurrence.[52] DDLPS, known for its high invasiveness, presents a high recurrence rate and a 5-year survival rate of 30%.[53] WDLPS and DDLPS exhibit low sensitivity to chemotherapy and radiotherapy. Hence, targeted therapy is preferred for these two subtypes of LPS.[2,54] The local recurrence and metastasis rate of PLPS ranges from 30% to 50%,[55] and it is usually insensitive to chemotherapy and radiotherapy, with a mortality rate is up to 50%.[2,56] In summary, due to differences in clinical manifestation, drug sensitivity, and recurrence tendency, there is an urgent need for more effective treatment methods.[2,53,55] The treatment of advanced or metastatic LPS remains a challenge for researchers.[55] At present, the commonly used treatment for LPS includes local surgical resection combined with drug therapy (doxorubicin, ifosfamide, doxorubicin, etc.); yet, the response rate is usually low, and the duration of the response is usually short, which limits the efficacy.[57]

At present, there is a lack of studies on the expression and mechanism of LMNB2 in LPS. Previous studies have primarily focused on LMNB1, and changes in the expression level of *LMNB1* have been found to have different effects on different types of tumors.[11,58]

In this study, we evaluated the correlation between *LMNB2* expression and immune cell infiltration in LPS patients. The results revealed that the infiltration level of

"CD8+T cell" was higher in the low expression group of *LMNB2*. In addition, *LMNB2* showed strong correlations with immune stimulators (including ULBP1, TNFRSF14, CD40L, and ENTPD1) (Figure 7). These results offer valuable insights into relevant potential targets for future immune drug development.

Enrichment analysis of expression profile data of LPS patients indicated that high expression of *LMNB2* was consistent with the upregulation of *E2F7* and *E2F8* of E2F family members. The E2F family plays a crucial role in the regulation of cell proliferation, differentiation, and apoptosis[59] and is closely related to the occurrence of a variety of tumors, including cervical cancer,[60-62] prostate cancer,[7] and glioblastoma.[63] Conversely, low expression of *LMNB2* was consistent with the upregulation of CD27. CD27, on binding with CD70, obtains co-stimulatory signals that enhance the activation, survival, proliferation, and differentiation of T cells, thereby supporting the maintenance of host defense function.[64] Certain studies have indicated that patients with CD27 deficiency are significantly more susceptible to malignant tumors. Therefore, we speculate that the occurrence of LPS may also be related to the decrease of immune function resulting from the low expression of CD27.

In tumors, one reason for the failure of immune control is the inhibition of effective function in infiltrating T cells.[65] The upregulation of programmed cell death-1 (PD-1) on $CD8^+$ T cells has emerged as a primary marker of T cell dysfunction. The anti-human PD-1/PD ligand 1 (PD-L1) monoclonal antibody has witnessed widespread use in both clinical practice research on tumors and infectious diseases. At present, the combination of PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) with an immune checkpoint blocker has entered clinical practice for treating patients with sarcomas. Compared with immune checkpoint blockers alone, the response rate of patients receiving combined immune checkpoint blockers is significantly improved.[66] Therefore, for the treatment of LPS, combining PD-1 and CTLA-4 with an immune checkpoint blocker might offer a relatively reliable method. This study focused on *LMNB2*, exploring its function and pathways in regulating LPS progression.

As mentioned previously, the most enriched functions of LMNB2 in BPs are "aging" and "histone modification." The main function of LMNB2 is to maintain the integrity of the nucleoskeleton by affecting chromosome distribution, which is involved in cell proliferation and senescence.[67] Thus, we postulated that high expression of *LMNB2* is associated with the inhibition of LPS cell senescence. However, the specific pathways by which *LMNB2* mediate LPS tumor cell senescence are not known. It is widely acknowledged that a wide variety of histone modifications, including phosphorylation, citrullination and sumoylation, has been implicated in LPS.[68-71] Thus, we postulated that the effects of histone modifications related to LMNB2 on LPS might be correlated with LPS progression and act as a promoting factor to the development of LPS.

5. Conclusion

Our study has identified elevated *LMNB2* expression levels as a risk factor for the poor prognosis of LPS patients. These findings contribute to a better understanding of the mechanism of LPS progression, suggesting that *LMNB2* could serve as a promising biomarker and potential therapeutic target for clinical treatment of LPS.

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Conflict of interest

The authors declare no conflicts of interest.

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Writing—original draft: Xinyu Li, Jialin Wu, Yang An
Writing—review & editing: Yang An.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Publicly available datasets were analyzed in this study. The datasets analyzed for this study can be found in the TCGA database (https://www.cancer.gov/about-nci/organization/ccg/research/structuralgenomics/tcga) and GEO database (https://www.ncbi.nlm.nih.gov/gds/?term=).

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

SHORT COMMUNICATION

On the *in silico* application of the center-of-mass distance method

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Abstract

This study aims to protocolize the utilization of the center-of-mass (CoM) distance method in GROMACS MD simulation software as a useful method for evaluating the binding affinity change in heterodimeric protein due to induced changes in one of the units. The hypothesis underlines the basic principles in biophysics, that an increase of the binding affinity is expected to reduce the relative CoM distance between monomers, while the opposite is expected to increase the relative CoM distance. However, it has been found that the CoM distance analysis must be strictly preformed during the convergent phase of systems' dynamics, once the monomers enter mutually stable conformation — a limitation which has usually been overlooked. The method was used to study the impact of *K417Y* severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) surface glycoprotein (S-protein) mutation. It has been found that the *K417Y* mutation favors reduced binding affinity between SARS-CoV-2 S-protein and human angiotensin-converting enzyme 2 (hACE2) receptor, which is due to the loss of the permanent K417-D30 salt bridge in favor of a temporary Y417-D30 hydrogen bond. The destabilizing impact of *K417Y* mutation on S-protein–hACE2 complex was confirmed by radius of gyration analysis.

Keywords: GROMACS; Simulation; Center-of-mass; Distance; *K417Y* mutation; SARS-CoV-2

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1. Introduction

The center-of-mass (CoM) distance method can be formally defined as a time continuous analysis of the distance between the CoM of two structures, which constitute together a common system of interest, such as monomers in heterodimer,[1] protein-ligand complex,[2] or a pair of key residues.[3]

The core definition of the CoM distance method has been applied in several *in silico* studies.[2-5] Ibrahim *et al.*,[2] compared inhibitor activity of erylosides B and lopinavir against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) main protease, using molecular dynamics (MD) simulations in addition to generalized born surface area binding energy calculations. MM/GBSA (molecular mechanics with generalized Born and surface area solvation) is used as a method to snapshot the free energy of the biding between ligands (erylosides B and lopinavir, specifically) to the SARS-CoV-2 main protease, which involves MD simulations with an explicit water solvent of the protein-ligand complex.[6] One of the methods used to show that erylosides B exhibits higher inhibitor activity against SARS-CoV-2 main protease than lopinavir is the CoM

distance. The conclusion was derived by measuring the average of the CoM distance between the ligands and SARS-CoV-2 main protease, during the course of 100-ns MD simulation. The average of the CoM distance for erylosides B was less than the average CoM distance for lopinavir, which was taken as a clue that erylosides B binds the SARS-CoV-2 main protease more effectively than lopinavir, demonstrating higher inhibitory potential.

Kumar *et al.*[3] analyzed pi-stacking interaction between Omicron receptor binding domain (RBD) Tyr501 and human angiotensin-converting enzyme 2 (hACE2) Tyr41, as N501Y is one of the key mutations responsible for increased infectivity.[1,7,8] The CoM distance between the aromatic rings has been measured, which in the case of pi-stacking interaction should not exceed 5 Å.[9] Such interaction is going to increase hACE2–surface glycoprotein (S-protein) binding affinity that will ultimately increase infectivity. The average CoM distance between aromatic rings of <5 Å in most of the conformations confirmed pi-stacking interaction between hACE2 Tyr41 and Omicron RBD Tyr501, which has been regarded as one of the reasons that explains the higher viral load and infectivity of the N501Y-bearing SARS-CoV-2 variant.

Apart from the CoM distance method, which is the main point of discussion in this study, researchers have also exploited additional *in silico* methods, such as residue fluctuation, radius of gyration, solvent accessible surface area and free energy landscapes, in order to analyze the fundamental properties of SARS-CoV-2 proteins' interactions[10,11] or evaluate the structural impact of selected mutations.[12-14] Docking studies proved to be especially useful in selecting highly effective SARS-CoV-2 inhibitors. Stability of the formed complexes has been used as a prime criterion to evaluate the inhibitory potential of each candidate. Ahamad *et al.*[10] found that anidulafungin has the same neutralizing capacity as the well-studied lopinavir. In another study, Ahamad *et al.*[11] evaluated the efficiency of several N-protein targeted antagonists and suggested 4E1RCat and TMCB as candidate drugs. MD simulations have also been used to study the impact of selected mutations. *In silico* findings that RBD and Heptad Repeat 1 mutations can impose major structural destabilization, affecting pre-binding protein structure, which may negatively impact current therapeutic efforts, have been presented in several papers.[12-14]

In some instances, using the average of the intermolecular CoM distance during the whole MD simulation,[2,3] may give rise wrong conclusions related to the binding affinity change. The reason for this is the fact that molecules usually exhibit sudden and sharp local and global movements, one relative to another, before entering the convergent state. In such cases, prior-convergent oscillations may easily suppress the impact of relative flat and convergent CoM distance amplitudes. Having them averaged during the course of the whole MD simulation, a wrong conclusion about the real direction of the affinity change may be derived.

To explain this situation better, let's take two random systems A and B, with a total of eight CoM distance snapshots [nm], such as the first five are prior-convergent samples and the last three convergent: A={4; 4.5; 3.9; 3.8; 3.5; 2.8; 2.75; 2.75} and B={3; 4; 4.5; 3; 3.2; 2.83; 2.82; 2.81}. By considering the integral systems' dynamics, upon the eight CoM distance snapshots, the average CoM distance in the system A is higher than the one in the system B (3.5 nm vs. 3.27 nm), which is interpreted in terms of reduced binding affinity between the units in system A in comparison to the binding affinity between the units in system B. However, the opposite of the previous conclusion is actually true, as the average CoM distance throughout the convergent state upon the last three snapshots in the system A is less than the one in system B (2.77 nm versus 2.82 nm).

An intuitive solution to this problem is to compute the average of the intermolecular CoM distance during the convergent phase only and neglect the non-convergent system's behavior. It is of crucial importance in the cases where the individual impact of a particular mutation or a combination of a few mutations need to estimated, a situation in which the wild-type and mutant complex share very similar CoM distance plots.

2. Method overview

Let W be the wild-type heterodimer and $\boldsymbol{M}$ the mutant of W. W can be retrieved from the Protein Data Bank in.pdb format. Molecular visualization software, such as PyMol, can be used to mutate the wild-type heterodimer W to M. Initially, both systems, W and M, need to be well-prepared and energetically optimized within self and toward the solvent that will guarantee that the systems are stable enough to undergo the process of MD simulation. The details of systems' preparation steps are described in the section 3. The method is formally described in the next section (Figure 1).

2.1. Formal description of the method

The CoM of a monomer of n atoms at positions r_j and masses: $m_1, m_2, \ldots, m_n$ can be computed as:

$$com = \frac{\sum_{i=1}^{n} m_i r_i}{\sum_{i=1}^{n} m_i} \quad \text{(I)}$$

where *com* is an oscillating point in the time (t): com(t)=(x(t),y(t),z(t),z(t)).

Equation I can be applied to compute monomer [1] (M_1) and monomer[2] (M_2)CoM:
$com_{M_1}(t) = (x_{M_1}(t), y_{M_1}(t), z_{M_1}(t))$ and
$com_{M2}(t) = ((x_{M_2}(t), y_{M_2}(t), z_{M_2}(t))$. Equation II can be used to compute the CoM distance between monomers M_1 and M_2 during the course of MD simulation.

$$d_{com}(t) = d_{M1,M2}(t) = \begin{pmatrix} (x_{M_1}(t) - x_{M_2}(t))^2 + (y_{M_1}(t) \\ -y_{M_2}(t))^2 + (z_{M_1}(t) - z_{M_2}(t))^2 \end{pmatrix}^{\frac{1}{2}} \quad \text{(II)}$$

For the sake of simplicity, we use $d_{com,W}$(t) and $d_{com,M}$(t) to denote the CoM distance between monomers in the wild-type and mutant heterodimer.

At first, we use d_{com}(t) to distinguish between non-convergent and convergent system's dynamics. During the non-convergent phase, monomers have not yet entered a mutually stable conformation, and large movements are likely to occur that will result in sharp d_{com}(t) oscillations. On the other hand, once they have entered a mutually stable conformation, the CoM distance is preserved at a relatively constant level, resulting in smooth d_{com}(t) transitions.

Given that t_{eq} is the earliest time point that marks the joint beginning of the convergent phase in both heterodimers, we compare average($d_{com,M}(t \geq t_{eq.})$) against average($d_{com,W}(t \geq t_{eq.})$), in order to draw a conclusion about the binding affinity change in comparative context, based on the fulfillment of condition (a), (b), or (c):

(a) If average($d_{com,M}(t \geq t_{eq.})$)>average($d_{com,W}(t \geq t_{eq.})$): Induced mutation(s) decrease intermolecular biding affinity;
(b) If average($d_{com,M}(t \geq t_{eq.})$)<average($d_{com,W}(t \geq t_{eq.})$): Induced mutation(s) increase intermolecular biding affinity;
(c) If average($d_{com,M}(t \geq t_{eq.})$)≈average($d_{com,W}(t \geq t_{eq.})$): Induced mutation(s) do not substantially alter intermolecular binding affinity.

2.2. Method implementation in GROMCAS MD simulation software

The method can be implemented in GROMACS MD simulation software, using the following output files:

(a) *.xtc* file: compressed MD trajectory file;
(b) *.tpr* file: portable binary run input file that contains the initial structure, the topology and simulation parameters;
(c) *.gro* file: that contains molecular structure in Gromos87 file format.

The first step is to call gmx make_ndx program to create separate index groups for the monomers in each heterodimer. The program reads the complete heterodimer structure, provided by.gro file. For each monomer in the system, separate index files can be compiled, provided by the residues' range selection option ri, such as: ri 1-597 (for monomer M_1) and ri 598-791 (for monomer M_2). The program generates.ndx file, followed by -o output flag. Typical command use would be:

gmx make_ndx -f npt.gro -o index.ndx

Having split monomers into separate index groups (by default indexed as groups 18 and 19), gmx distance program can be used to compute the distance between monomers' CoM, during the course of the simulation. The following command computes and writes down the distance between monomers' CoM in.xvg file, having provided.xtc and.tpr files as input arguments and having selected corresponding monomers from the.ndx file:

gmx distance -f md.xtc -s md.tpr -n index.ndx -oall output_file.xvg -select 'com of group 18 plus com of group 19'

The obtained CoM distance results in.xvg format: $d_{com,W}$ (for the wild-type heterodimer) and $d_{com,M}$ (mutant heterodimer) that can be plotted in MS Excel. One can use the plot to identify the earliest time point $t_{eq.}$, when both heterodimers enter stable conformation. We can identify the binding affinity impact of induced protein mutations, depending on which of the conditions (a), (b), or (c) becomes true.

3. *In silico* experiment: Systems preparation and MD simulation

For the purpose of the experiment, Protein Data Bank (https://www.rcsb.org) structure: 6M0J,[15] was used as a wild-type molecular complex. 6M0J heterodimer (https://www.rcsb.org/structure/6m0j) includes two monomers, namely, chain A (hACE2 receptor, residues range: [19 – 615]) and chain E (SARS-CoV-2 S-protein RBD, residues range: [333 – 526]); N-acetyl-D-glucosamine ligands; and additional metal ions, such as zinc cations. Amino acids included in the 6M0J model represent the key interface of hACE2-RBD interactions.

PyMol software (https://pymol.org/2/, version 2.5.4) was used to clean up all non-protein content and mutate wild-type K417 (Lys417) in SARS-CoV-2 S-protein to Y417 (Tyr417). In spite of 3D molecule visualization, PyMol also enables easy content modification. PyMol mutagenesis tool was used to mutate wild-type K417 (Lys417) to Y417 (Tyr417) in the SARS-CoV-2 S-protein.

Both heterodimers, bearing K417/Y417 in the S-protein, followed equal preparation procedure. Heterodimers were dissolved under the SPC/E (simple point-charge/extended)

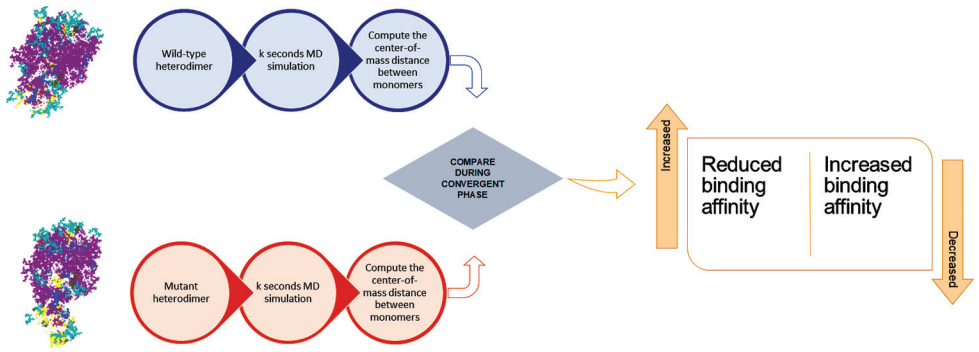

Figure 1. Visualization of the proposed method.

water model, having placed them and centered into a cubic box at 1 nm minimum distance from the edge of the box. Brooks *et al.*[16] showed that Charmm27 all-atom force field was used for the purpose of simulation. Totally, 25 water molecules were substituted with 25 Na^+ ions to bring up the systems to the neutral net charge. The systems were relaxed and optimized within-self applying the steepest descent energy minimization algorithm,[17] until potential energy $E_{pot} < -10^5\, kJmol^{-1}$.

The purpose of 100-ps NVT equilibrium phase, controlled by V-rescale thermostat, was to bring the systems under the desired temperature of 310 K. V-rescale belongs to a sophisticated group of algorithms named thermostats and its role is to maintain a constant temperature level in the system throughout the process of MD simulation. The NVT equilibrium phase of 100-ps granted referent coupling pressure of 1 bar, assuming water isothermal compressibility equivalent to $4.45\times10^{-5} bar^{-1}$ at $T = 310\ K$. Relaxed heterodimers were subjects to 50-ns MD simulation in GROMACS software.[18]

The aim of the *in silico* experiment was to evaluate the relative binding affinity change due to *K417Y* mutation in a comparative context: increased, decreased or no change, by measuring the CoM distance between the monomers in the common convergent state.

4. Results

Figure 2 shows the CoM distance between monomers in K417/Y417 heterodimers: $d_{com,K417}(t)$ and $d_{com,Y417}(t)$, during the course of 50-ns MD simulation. The substitution of positively charged Lysine(k) to polar, uncharged Tyrosine(y) at position 417 in the S-protein of SARS-CoV-2 may increase, decrease or have no substantial effect on S-protein–hACE2 binding affinity.

Following the method's considerations, we should first identify the beginning of the convergent state ($t_{eq.}$). Both heterodimers, K417 (wild-type) and Y417 (mutant), enter relatively stable CoM distance amplitudes after 46.7 ns

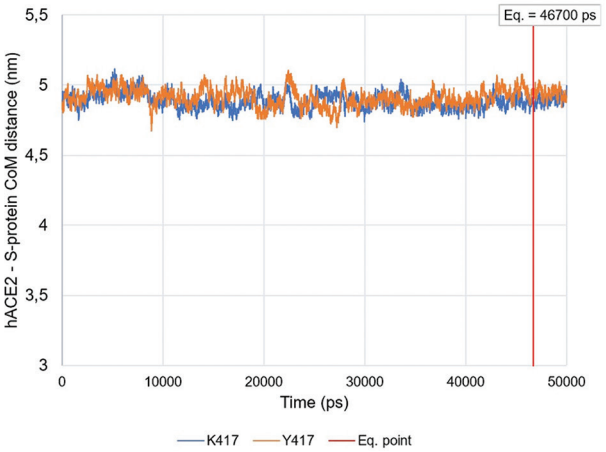

Figure 2. Evaluation of the impact of *K417Y* mutation by the means of $d_{com}(t)$ method.

(Figure 2), which is taken an as equilibrium point, $t_{eq.}$= 46.7 ns. During the convergent phase t = [46.7–50] ns, $d_{com,K417}(t)$ and $d_{com,Y417}(t)$ range <0.2 nm (Figure 2 and Table 1).

Throughout the convergent phase [46.7–50] ns, the mutant heterodimer Y417 exhibits higher intermolecular CoM distance than K417 wild-type: $d_{com,K417}$ = 4.943302115±0.037474346 nm versus $d_{com,K417}(t)$ = 4.89718429±0.033584437 nm (Figure 2 and Table 1). The increase of the CoM distance in Y417 heterodimer relative to K417 favors partially reduced S-protein–hACE2 binding affinity in 6M0J heterodimer specifically.

Strictly speaking, and methodologically, condition (a) is fulfilled: $average(d_{com,Y417}(t \geq 46.7\ ns))$ = 4.943302115 nm > 4.89718429 nm = $average(d_{com,K417}(t \geq 46.7\ ns))$ (Table 1), and the corresponding conclusion for partially reduced binding affinity between the monomers due to *K417Y* mutation is derived.

A key point in addition to the obtained results and derived conclusion is the fact that the analysis was

performed during the common convergent state in both heterodimers and not throughout the course of the entire simulation [0 – 50] ns, resembling the protocols in previous studies,[2,3] potentially resulting in misleading conclusion related to the binding affinity change.

5. Discussion

Non-covalent interactions, specifically involving K417/Y417 residues, were analyzed, based on the relaxed, crystal pose K417/Y417 PDB structures, using the Ring 3.0 server[19] (https://ring.biocomputingup.it/), for the following cutoff values: maximum ionic bond distance 4 Å, maximum hydrogen bond donor-acceptor distance 3.5 Å, maximum π–π stacking distance 4 Å, and Van der Walls radius intersection fraction of <0.01 Å.

It has been found that S-protein K417 participates in two interactions with hACE2 D30: an ionic bond/salt bridge and a hydrogen bond (Figure 3). Inside the S-protein, K417 forms two additional hydrogen bonds (K417-L455, K417-N422), and three van der Walls interactions of minor electrostatic impact involving D420, N422, and L455 (Figure 3). On the other hand, it has been found the S-protein Y417 forms only one hydrogen bond and two Van der Walls contacts with hACE2 D30 (Figure 3). Inside the S-protein, there is a hydrogen bond: Y417-N422 and Y417-D420 Van der Walls contact (Figure 3).

Residue interactions analysis in Ring 3.0 server[19] showed that the major change, which happens due to the *K417Y* mutation, is the alteration of the much stronger salt bridge to a hydrogen bond, suggesting this change as a major point of interest, which has been analyzed in terms of 50-ns MD simulation (Figure 4 and Table 2).

Table 1. Analysis of $d_{com,\ K417}$ versus $d_{com,\ Y417}$ during the convergent phase [46.7–50] *ns*

Heterodimer	Average CoM distance (nm)	St. dev. CoM distance (nm)	Range (nm)
K417 (wild-type)	4.89718429	0.033584437	0.185
Y417 (mutant)	4.943302115	0.037474346	0.197

Abbreviations: st. dev.: Standard deviation; CoM: Center-of-mass.

In K417 heterodimer, the salt bridge was formed between deprotonated carboxylic acid COO^- in D30 (aspartic acid, hACE2) and the positively charged ε-amino group NH_3^+ in K417 (Lysine, S-protein) (Figure 4). The salt bridge was changed to hydrogen bond in *Y417* mutant, formed between D30 carboxylate ion and K417 phenolic hydroxyl group (-OH) (Figure 4).

Although the salt bridge is a k-fold stronger interaction than the hydrogen bond, it has been inspected for the occupancy of these interactions, as the overall impact of a strong but temporary interaction may be outcompeted by a weaker but permanent interaction(s).

Figure 4 shows the occupancy of D30-K417 salt bridge and D30-Y417 hydrogen bond per frame, during the course of MD simulation [0 – 50] ns. Binary coding scheme "1/0" is used to denote the presence/absence of a specific interaction, "1" for present and "0" for absent interaction (Figure 4). The salt bridge is present, if the distance between COO^- (D30) and NH_3^+ (K417) is <0.4 nm.[20] The module gmx distance was used to calculate the distance between COO^- (D30) and NH_3^+ (K417) per frame. The presence of the hydrogen bond was detected based on the geometric criteria for hydrogen bond formation: donor-acceptor distance (r_{DA}) <0.35 nm and hydrogen-donor-acceptor angle (∢had) <30.[21] The module gmx hbond was used for this purpose.

Table 2 summarizes the occupancy of the D30-K417 salt bridge and D30-Y417 hydrogen bond, during the course of the MD simulation t = [0–50] *ns* and specifically during the convergent phase, $t \geq 46.7$ *ns*. In both cases, the occupancy of the salt bridge was higher than the occupancy of the hydrogen bond (Figure 4 and Table 2).

During the convergent phase ($t \geq 46.7$ *ns*) or the stabilized systems' dynamics, the salt bridge becomes a permanent intermolecular interaction with an occupancy = 98.5% (Table 2 and Figure 4), while the hydrogen bond shifts

Figure 3. K417/Y417 contacts analysis in RING 3.0. Indicators: red dashes denote salt bridge; blue dashes denote hydrogen bond; blue-gray dashes denote Van der Walls contacts.

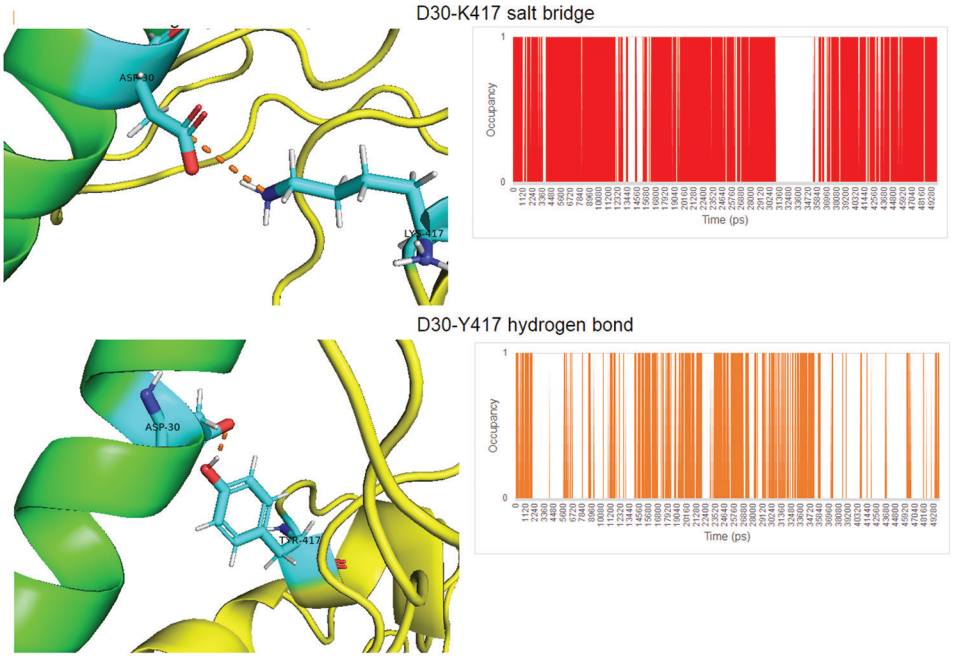

Figure 4. Visualization of mutation specific non-covalent interactions and their occupancy [0–50] ns.

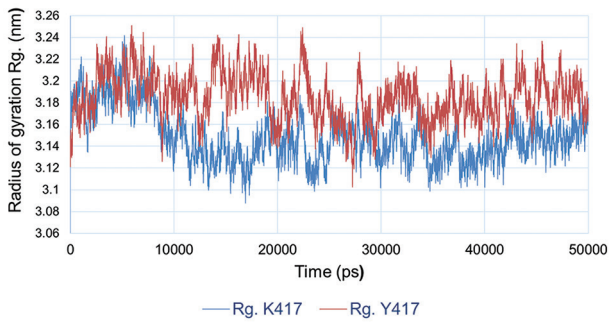

Figure 5. Radius of gyration.

to an interaction of a temporary character with an occupancy = 24.8% (Table 2 and Figure 4). The change of the strong and permanent salt bridge in *K417* wild-type heterodimer to a temporary hydrogen bond in *Y417* mutant, during the convergent phase, favors partial decrease of the binding affinity between SARS-CoV-2 S-protein and hACE2.

This conclusion is the same as the conclusion derived by the application of the CoM distance method, confirming the reliability of the proposed methodology.

The reduced binding affinity due to K417Y substitution will also favor minor complex destabilization, which has been proved in terms of the increased radius of gyration (Table 2 and Figure 5). The average radius of gyration in Y417 complex equals to 3.1852 ± 0.0217 nm, compared to 3.1488 ± 0.0242 nm in K417 (Table 2 and Figure 5).

Table 2. Occupancy of mutation-specific interactions in heterodimers and radius of gyration analysis

Heterodimer	*K417* (wild-type)		*Y417* (mutant)	
Occupancy [0–50] ns	D30-K417 salt bridge	77.9%	D30-Y417 hydrogen bond	41.9%
Occupancy [46.7–50] ns		98.5%		24.8%
Radius of gyration (average±standard deviation)	3.1488±0.0242 nm		3.1852±0.0217 nm	

Rather than evaluating the strict impact of *K417Y* mutation, which may require MD simulation longer than 50 ns, the experiment, experimental design and results reported in this study serve the illustrative purpose for accurate application of the CoM distance method.

Even though both systems enter a steady state after 46.7 ns or a total of 3.3 ns of assumed convergent system behavior have been observed, monitoring a much longer convergent state would be a better guarantee for not being trapped in a local well.

6. Conclusion

This study depicts the application of the CoM distance method. The CoM distance analysis should be limited to the common convergent state in both systems, which guarantees accurate affinity analysis. The application of the method can be further expanded to artificial intelligence-based protein structure databases, complexes modeled

with protein-protein docking, and affinity testing using the proposed method.

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Availability of data

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

CASE REPORT

Hereditary angioedema: A case report

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Abstract

Angioneurotic edema (ANE) is a frequently encountered presentation in the emergency department. This condition manifests itself as sudden, unpredictable episodes of edema in cutaneous and mucosal tissues, commonly affecting the eyes, oral cavity, lips, and larynx. It is crucial to acknowledge that ANE is a component of a range of allergic manifestations, often associated with urticaria and occasionally of non-allergic origin. In some cases, this condition can lead to laryngeal edema, causing airway obstruction and potentially fatal consequences if not diagnosed in a timely manner. After 2007, the term ANE was replaced by the term angioedema (AE) in the literature for its conciseness and wider recognition. The purpose of this comprehensive article is to present a detailed analysis of a clinical case involving a 75-year-old patient diagnosed with AE, accompanied by a thorough examination of relevant literature that has significantly contributed to our understanding of this complex medical condition.

Keywords: Angioedema; Larynx; Facial; Treatment

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1. Introduction

Angioneurotic edema (ANE), also known as Quincke's disease, is a medical condition that poses a potential threat to one's life. It is characterized by localized swelling in the subcutaneous and submucosal tissues of both the upper respiratory and gastrointestinal tracts.[1] After 2007, the term ANE was replaced by the term angioedema (AE) in the literature for its conciseness and wider recognition.[2] It is important to note that AE can be categorized into two distinct types: allergic AE and non-allergic AE.[3] Furthermore, non-allergic AE may be further subdivided into various subtypes, such as hereditary AE (HAE), acquired AE (AAE), drug-induced AE, and idiopathic AE. Hereditary angioedema is a condition that can be inherited and is caused by mutations in genes that affect the production of C1-inhibitor.[3,4] In the United States, ANE has a lifetime prevalence rate of approximately 25%, resulting in over one million emergency department visits on a yearly basis.[5] It is worth mentioning that the majority of cases (approximately 90%) can be attributed to allergic reactions, which are characterized by a rapid type 1 hypersensitivity response that activates mast cells and basophils. On the contrary, non-allergic AE, mediated by bradykinin inhibition, often presents with a delayed onset, taking hours to days to manifest after exposure, and in some cases, it may even take several months to become apparent.[6] The excessive production of bradykinin, a potent vasodilatory mediator, contributes to the swelling of the mucosa

and submucosal tissues.[6] During an AE episode, plasma levels of bradykinin significantly increase, reaching up to seven times the normal level.[7] Unlike histamine-induced AE, antihistamines are ineffective when bradykinin is the causative factor.[7,8]

2. Case presentation

We report the case of a 75-year-old patient with a personal history of recurring episodes of facial edema since a young age and a family history of AE in a parent and brother. The patient was admitted to the emergency room with generalized edema of the face, which developed over 14 h without dyspnea or other associated signs. During the clinical examination, we observed a eupneic patient with edema affecting the periorbital, lip, and jugular regions (Figure 1A-C). The floor of the mouth and tongue was spared. Nasofibroscopy revealed a normal-looking, mobile larynx. The patient was treated with high-dose corticosteroids and oxygen therapy. An etiological assessment was subsequently performed to confirm the C1-inhibitor deficit.

3. Discussion

Angioedema is a rare genetic disorder caused by a deficiency in the C1-inhibitor. The first onset of symptoms typically occurs during childhood, with the frequency of attacks increasing around puberty. The skin, upper respiratory tract, and gastrointestinal tract are commonly affected in 98% of the cases.[9]

3.1. Diagnosis

The diagnosis of AE is significantly delayed, with an average delay of 8.5 years worldwide, mainly due to the lack of awareness of the disease.[10,11] This delay is aggravated by the fact that AE symptoms, such as abdominal pain, can overlap with other conditions and gastroenterological diseases, leading to misdiagnosis.[12] Furthermore, individuals may not be screened for the disease following initial symptoms, especially if they do not have family members with a confirmed diagnosis of AE.

Detailed history taking is crucial in the evaluation of AE, encompassing inquiries about familial and drug backgrounds. Patients with AE manifest swelling in areas such as the throat, face, lips, mouth, eyes, genitalia, and extremities.[9] Furthermore, people with abdominal AE may display symptoms such as colicky abdominal pain, vomiting, and abdominal distension as a result of submucosal edema of the bowel wall, which can lead to intestinal blockage. Swelling of the uvula or tongue can be directly observed; however, it is advisable to perform a laryngoscopy to evaluate the condition of the vocal cords. Concerns regarding the airway should be addressed when patients present with wheezing, stridor, voice hoarseness, or breathing difficulties. The initial step in airway assessment is to evaluate airway patency. During allergic AE episodes, individuals experience pruritic urticaria, and sometimes, non-pitting edema may be observed in the extremities.[9]

Routine laboratory values in patients with HAE are typically within the normal range. However, if there are clinical indications or imaging studies suggestive of HAE, serum complement screening should be performed. This screening often reveals low levels of C4 and CH50, whereas C3 levels remain normal. A confirmed diagnosis of HAE necessitates the measurement of reduced C1-inhibitor function levels.[9]

3.2. Treatment

Angioedema should be treated as early as possible. Some patients with HAE may require airway intervention, such as intubation. Treating an acute HAE attack in its early stages through self-administration serves as a preventative measure

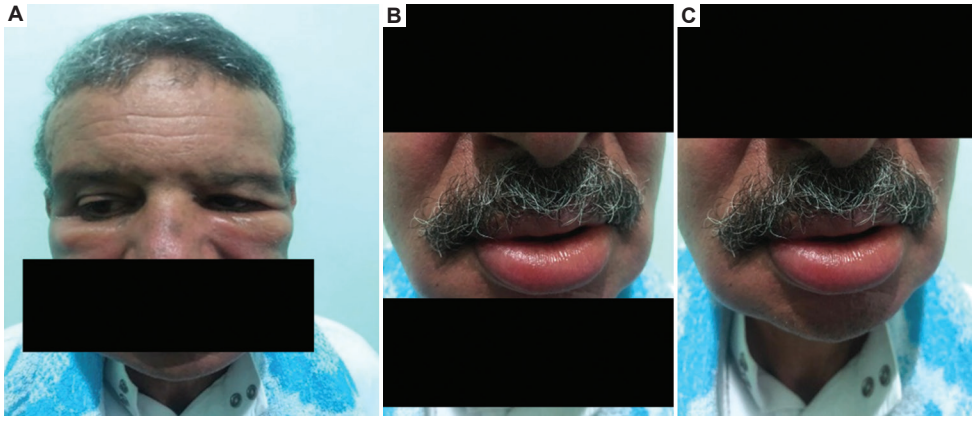

Figure 1. Clinical examination reveals an eupneic patient with edema affecting various facial regions: (A) periorbital region; (B) lip region; (C) jugular region.

against the development of severe complications. This, in turn, facilitates prompt recovery and reduces the duration of painful episodes experienced during the attacks.[13]

The therapeutic options for the treatment of HAE have expanded significantly in recent years, offering both long-term prophylactic strategies aimed at preventing HAE attacks and acute treatment options to address immediate symptoms. Historically, attenuated androgens have been utilized for HAE prophylaxis; however, it is crucial to note that these medications can potentially lead to severe adverse effects. Thus, although they have proven effective in some cases, their side effects necessitate careful consideration. There are several noteworthy options for acute treatment. One option is Berinert®, which functions as a nanofiltered C1-inhibitor (human) replacement product. Another choice is Firazyr® (icatibant), an antagonist of the bradykinin B2 receptor that plays a role in the development of AE. Finally, Kalbitor® (ecallantide) is a plasma kallikrein inhibitor that can be employed to manage acute HAE attacks. Each of these treatment modalities offers distinct mechanisms of action and may be suitable for different patients, depending on their individual requirements. The availability of these diverse therapeutic options represents a significant advancement in the field of HAE management and provides health-care professionals with a broader range of tools to effectively address this condition.[13]

3.3. Prophylaxis

It is essential to administer short-term prophylaxis before high-risk procedures to patients at risk.[13] Anesthesiologists should possess comprehensive knowledge of the guidelines for HAE and AAE.[2,14-16] Patients with HAE should consider short-term prophylaxis, such as plasma-derived C1-inhibitor concentrate and a short-term course of high-dose therapy with attenuated androgens, or fresh frozen plasma, to minimize the risk of attacks triggered by specific factors, such as invasive medical procedures.[17]

Long-term prophylactic therapy aims to minimize the occurrence and severity of seizures and prevent progression to emergencies or hospitalization in patients with HAE. The primary objective is to enhance the overall well-being of patients. The risk–benefit ratio of such treatments should be carefully considered for each patient, considering their adverse effects, administration method, and cost. Prolonged prophylaxis is typically recommended for patients experiencing frequent (defined as more than 24 symptomatic days/year) or severe (at least one severe attack/month) AE attacks, those with a history of laryngeal edema, significant school or occupational absences, a marked decline in quality of life, or patients who are unable to manage the treatment of severe crises.[18,19] Oral medications, such as danazol and tranexamic acid, continue to be the most commonly prescribed medications. However, patients should undergo regular re-evaluation and discussion with their health-care provider during each consultation to determine whether prophylactic treatment is still necessary.

4. Conclusion

Angioedema is an uncommon pathological condition characterized by sudden episodes of swelling under the skin and mucous membranes and can vary widely in the age of onset among individuals. Early detection is crucial because of the considerable morbidity associated with laryngeal swelling. The clinical presentation characterized by skin and mucous membrane swelling, minimal itching, recurring episodes, absence of hives, and unresponsiveness to antiallergic treatment, along with a familial history of similar cases, should guide the diagnosis. Upon strong clinical suspicion, confirmation can be obtained through laboratory tests involving the measurement of C4- and C1-inhibitor levels. Consequently, clinicians must closely collaborate with a team of experienced biologists to study this condition. The significant advancement in genetic research holds promise for enhancing our understanding of the diverse range of clinical manifestations. However, the current therapeutic interventions are suboptimal because they often have notable adverse effects.

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Ethics approval and consent to participate

The patient provided verbal consent for this case report.

Consent for publication

Verbal consent was obtained from the patient for publication.

Availability of data

The data used in this study are available from the corresponding author upon request.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

COMMENTARY

Identification of stress-induced epigenetic methylation onto dopamine D2 gene and neurological and behavioral consequences

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Abstract

The D2 dopamine receptor (*DRD2*) gene has garnered substantial attention as one of the most extensively studied genes across various neuropsychiatric disorders. Since its initial association with severe alcoholism in 1990, particularly through the identification of the *DRD2 Taq A1* allele, numerous international investigations have been conducted to elucidate its role in different conditions. As of February 22, 2024, there are 5485 articles focusing on the *DRD2* gene listed in PUBMED. There have been 120 meta-analyses with mixed results. In our opinion, the primary cause of negative reports regarding the association of various *DRD2* gene polymorphisms is the inadequate screening of controls, not adequately eliminating many hidden reward deficiency syndrome behaviors. Moreover, pleiotropic effects of *DRD2* variants have been identified in neuropsychologic, neurophysiologic, stress response, social stress defeat, maternal deprivation, and gambling disorder, with epigenetic DNA methylation and histone post-translational negative methylation identified as discussed in this article. There are 70 articles listed in PUBMED for DNA methylation and 20 articles listed for histone methylation as of October 19, 2022. For this commentary, we did not denote DNA and/or histone methylation; instead, we provided a brief summary based on behavioral effects. Based on the fact that Blum and Noble characterized the *DRD2 Taq A1* allele as a generalized reward gene and not necessarily specific alcoholism, it now behooves the field to find ways to either use effector moieties to edit the neuroepigenetic insults or possibly harness the idea of potentially removing negative mRNA-reduced expression by inducing "dopamine homeostasis."

Keywords: Dopamine D2 receptor; Epigenetic modification; Neurological disorders; Stress

1. Introduction

In 2013, Blum's research team initiated an inquiry into the motivations behind alcohol consumption and high rates of drug-seeking behaviors, particularly in response to stressful situations.[1-4] Their goal was to raise awareness and understanding of the underlying mechanisms driving such behaviors. Moreover, they posed additional questions regarding the millions of individuals who seek out and engage in high-risk novelty situations, pondering the consequences of pleasure-seeking behaviors. They suggested that the answers to these questions may lie within the intricate workings of our brains and perhaps within our genetic makeup.[5,6]

America is currently facing the worst opioid epidemic in history. In 2021, the centers for disease control and prevention (CDC) reported 106,699 overdose deaths in the US, with 80,441 of those deaths related to opioids. In addition, provisional data from the CDC indicated that opioid deaths in 2022 would rise to approximately 82,998.[7,8]

Blum *et al.* established the concept of reward deficiency syndrome (RDS) in 1995 as a potential predictor of addictive and impulsive behaviors.[5,9-11] This theory has since been supported by numerous studies, indicating that behavioral, cognitive, and emotional disturbances observed in psychiatric disorders, including RDS, are linked to functional deficits in neurological pathways.[12-17] For instance, the D2 dopamine receptor (*DRD2*) *Taq A1* allele has been consistently associated with numerous behavioral phenotypes, including aggression,[18] alcoholism, addictive behaviors,[19] and neuropsychiatric disorders. Both

the *DRD1* and *DRD2* genes are linked to reward pathways and mechanisms.[20] The culmination of biochemical processes in mesolimbic regions leads to rewarding phenomena, particularly in the nucleus accumbens (NAc), where increased dopamine levels in synaptic spaces interact with *DRD1*, *DRD2*, and other receptor subtypes.[21,22] Positron emission tomography studies have provided further insights, demonstrating lower *DRD2* availability in individuals with dependence on cannabis, psychostimulants, opioids, or alcohol, as well as those who are obese, compared to control subjects.[23-28]

Furthermore, studies have revealed an association between the A1 and B1 minor alleles of the *DRD2* gene and cocaine use disorder (CUD).[26] These findings suggest that genetic variations in the *DRD2* gene, located on chromosome 11 at the q22-q23 region, contribute to an increased susceptibility to psychostimulant use disorder (PUD). Interestingly, these observations align with early research by Gold's group, which hinted at the potential therapeutic efficacy of bromocriptine, a D2 agonist, in combating cocaine abuse and dependence.[29] Unlike the *DRD2 Taq A1* allele, which is associated with decreased D2 receptor levels, the main variant, *DRD2 Taq A2* allele, is characterized by normal D2 receptor levels, possibly offering protection against psychostimulant misuse and abuse.[30]

Historically, Dackis and Gold were among the first to propose the use of the potent D2 agonist bromocriptine as an epigenetic therapy for severe cocaine dependence.[29] However, clinical trials revealed that bromocriptine led to the down-regulation of DRD2 receptors and did not prove to be effective for this purpose, resulting in its limited clinical utilization.[31]

This commentary has uncovered a plethora of studies delineating epigenetic alterations occurring within the *DRD2* gene across both substance-related and non-substance-related RDS behaviors.

2. Epigenetics and addictive behaviors

Epigenetics refers to the molecular modifications imposed on chromatin within the nucleus of a cell, playing a crucial role in regulating various DNA-related processes, including chromatin organization, DNA repair, RNA transcription, and splicing, among other essential cellular functions.[32] Substance use disorder (SUD) serves as a prime example of how environmental factors can influence gene expression.[33] In this regard, a person's experiences, particularly volitional repetitive drug use, can modify the epigenome in the brain in a way that is specific to certain brain regions and cell types.[34] It is hypothesized that dysregulation and modification of DNA-related processes induced by drugs can lead to epigenetic alterations, which may contribute to aberrant cellular functions that facilitate the pathogenesis of psychoactive substance dependence. Nestler's group has previously proposed that gaining insights into epigenetic processes holds therapeutic promise.[35] Targeting significant drug-induced epigenetic alterations within the brain could potentially disrupt the cycle of drug dependence, thereby preventing individuals from succumbing to a relentless cycle of addiction.[35]

Comprehending the intricacies of the neuroepigenetic landscape necessitates acknowledging the array of epigenetic modifications.[35] Among these, a significant epigenetic change occurring in adverse environments involves histone post-translational modifications (PTMs), such as methylation and even dopaminylation.[36,37] Essentially, chromatin consists of DNA that is intricately wound around histone protein octamers and forms nucleosomes, which subsequently allows for compact packaging within the cell nucleus. This structural arrangement serves as an adaptable scaffold that responds to external stimuli. Notably, histones are rich in arginine and lysine residues, contributing to their highly basic nature. PTMs of these residues and others on histone N-terminal tails, extending from the nucleosome core, modulate the physical properties and charge distribution of chromatin, thereby regulating DNA-associated processes.

Histone subunits undergo a multitude of PTMs, including but not limited to acetylation, methylation, phosphorylation, adenosine diphosphate ribosylation, ubiquitylation, and sumoylation, with an expanding array of newly identified modifications.[38,39] These modifications occur on over 50 distinct sites across histone proteins.[38,39]

According to Allis *et al.*, "histone PTMs are reversible; they are dynamically deposited by "writer" enzymes, recognized by "reader" proteins which mediate the cellular response, and removed by "eraser' enzymes."[36] Studies have demonstrated that the expression and activity of numerous writer, eraser, and reader proteins are dysregulated in both addicted individuals and animal models of addiction.[40-42] This dysregulation has spurred interest in the development of novel epigenetic therapies for addiction. The restoration of normal function to these proteins, either through small molecule interventions or functional food complexes that help rebalance neurotransmitter levels, such as dopamine, represents a promising avenue for anti-addiction epigenetic treatments.[43-52]

3. Epigenetic biomarkers

3.1. DNA methylation

A biomarker is a quantitative, measurable indicator of a biological molecule, state, or condition.[53] DNA methylation

is the most researched epigenetic biomarker due to its chemical stability, role in mammalian development and disease, and its significant role in modulating gene expression across a wide array of biological processes.[54,55] Although DNA methylation induces physical changes to gene structure, it is reversible in nature. Its primary function is to impede DNA transcription, thereby suppressing the expression of specific genes. DNA methylation tests have become more affordable and accessible and require only a small amount of DNA, which can be obtained from body fluids, cells, or tissues.[19] In addition, DNA can be isolated from bacteria, viruses, plants, or mammals. DNA methylation is a biochemical process characterized by the addition of a methyl group to DNA molecules. A common occurrence is the addition of a methyl group to the 5-carbon position of a cytosine ring, forming 5-methylcytosine (5-mC).[56] DNA methylation assays are techniques employed to quantify the levels of 5-mC within DNA samples. The enzyme DNA methyltransferase (DNMT) plays a pivotal role in catalyzing DNA methylation, particularly at CpG dinucleotide sites.[57] Notably, DNMT-1 is primarily responsible for DNA replication in the mitotic cells of the brain, which exhibit the highest levels of DNA methylation in the body.[58] DNMT-3A and DNMT-3B, on the other hand, regulate methylation patterns during early developmental stages.[59]

NA methylation plays a critical role in early brain development and the specification of regions through gene expression.[57] It also significantly influences mutational events associated with various cancers, increasing the risk of gene mutations and the inactivation of specific tumor-suppressor genes.[60-64] For instance, exposure to environmental carcinogens, such as pollution, can induce mutations in genes responsible for DNA methylation, leading to altered cellular states such as proliferation or differentiation and ultimately resulting in cancer.[64]

Moreover, DNA methylation is involved in genomic imprinting, a process where genes are silenced or inactivated through DNA methylation. Imprinting occurs when one allele from either the father or mother is silenced, resulting in an imprinted gene.[65] These parent-of-origin effects can be inherited by gametes and passed down to offspring, giving rise to various diseases such as Prader-Willi syndrome and Angelman syndrome.[66,67] This mode of inheritance also aids in understanding the role of DNA methylation in psychiatric disorders such as major depressive disorder, bipolar disorder, schizophrenia, autism, and related conditions. This finding is significant as it provides crucial insights into how abnormalities in this mechanism contribute to the pathophysiology of diverse disorders, and it suggests the potential of DNA methylation as a therapeutic target.[67]

3.2. DNA demethylation

DNA demethylation is a process that occurs alongside DNA methylation but is not as widely understood.[68] It serves as a biomarker for DNA damage, involving the removal of a methyl group from DNA, which can occur actively or passively in both dividing and non-dividing cells. Passive demethylation entails the loss of 5mC during DNA replication, while active demethylation involves the alteration or removal of a methyl group from 5 mC. Notably, 5 mC, a methylated form of cytosine, is commonly utilized as a point of interest for gene mutations and as an epigenetic marker due to its regulatory role in gene transcription.[55,69]

Furthermore, a derivative of 5mC known as 5-hydroxymethylcytosine (5hmC) is abundantly present in various organ tissues, particularly in the brain. DNA demethylation acts as a marker for DNA damage and facilitates repair processes by identifying potentially mutated sites.[70] Primordial germ cells of an embryo and developing zygotes are the primary sites where demethylation occurs, emphasizing its key role in differentiation mechanisms.[71]

DNA demethylation is mediated by enzymes from the ten-eleven translocation (TET) family.[72,73] The TET enzymes function as tumor suppressors in various malignancies, and their loss or dysfunction is closely associated with rapidly mutating cancers.[74] In addition, thymine DNA glycosylase plays a crucial role in DNA demethylation and normal development by initiating base excision repair, which is essential for repairing damaged DNA throughout the cell cycle.[75]

In summary, DNA demethylation serves as a mechanism for epigenetic reprogramming of genes, influenced by environmental risk factors such as injuries and substance use.

3.3. Histone modification

Histones, a class of proteins, facilitate the packaging of DNA into structural units known as nucleosomes. This packaging provides structural support and ensures that DNA fits appropriately within the nucleus of the cell. The modification of histones exemplifies epigenetic regulation, as it influences transcription and alters phenotypes in response to environmental stimuli and stressors. Histone modifications achieve this process by tightly compacting DNA, hindering its accessibility to the cellular machinery.[76] Conversely, histone relaxation facilitates increased access of proteins to DNA, thereby enhancing its susceptibility to analysis by the cell. Histone alterations have also been found to affect DNA repair and replication, in addition to cell state modifications.[77] They are also utilized to synthesize macromolecules like lipids and carbohydrates, as well as to regulate cell metabolism and energy outputs.[78]

It is crucial to note that histone alterations frequently have an indirect effect by activating downstream signaling cascades through proteins.

3.4. Histone acetylation

Histone acetylation represents a reversible epigenetic mechanism often associated with enhanced gene transcription and implicated in processes underlying memory formation and drug addiction. It serves as a conduit for environmental cues, particularly those from drugs of abuse, to elicit targeted modifications in gene expression. The enzymatic regulation of acetylation involves histone deacetylases (HDACs) and histone acetyltransferases (HATs).[79] During histone acetylation, a negatively charged acetyl group is affixed to lysine residues on histone proteins,[80] resulting in a relaxed chromatin structure conducive to transcription factor binding and heightened gene expression.

Among histones, H3 and H4 are highly conserved and have garnered significant attention due to their pivotal roles in chromatin organization.[81] Histone acetylation often occurs concomitantly with other chromatin modifications. Similar to DNA methylation and demethylation, histone acetylation is anticipated to function as a biomarker for various diseases. A reduction in histone acetylation has been associated with neurodevelopment disorders, neural degeneration, plasticity, and memory impairment. The CLOCK protein exemplifies HAT and stands as a prominent transcription factor integral to circadian rhythms and cellular homeostasis. Disruptions in this HAT cause a number of changes associated with sleep deprivation and bipolar disorder manic-like behaviors.[82] A thorough investigation into histones will be essential for the development of numerous therapeutic strategies for psychiatric disorders, neurodegenerative diseases, and tumor cells.

3.5. Chromatin remodeling

Changes to histone proteins can influence the structure of chromatin, encompassing modifications such as acetylation, deacetylation, methylation, and demethylation.[83] These modifications ultimately dictate whether transcription is activated or repressed. Chromatin that is tightly condensed and transcriptionally inactive is termed heterochromatin, where genes are typically silenced or inactivated. Conversely, euchromatin refers to loosely condensed chromatin that is more accessible for transcription. In euchromatin, DNA is readily accessible for binding by transcription factors and other DNA-binding proteins, facilitating the regulation of gene expression.[84] The process of transitioning chromatin from a condensed to a more accessible state is referred to as chromatin remodeling, as discussed in earlier sections detailing histone modifications.

Chromatin plays a pivotal role in a multitude of cellular functions, encompassing DNA repair, DNA replication, chromosome segregation, and signal transduction, among others. Chromatin remodelers typically consist of multiprotein complexes and enzymes that harness adenosine triphosphate hydrolysis to modify histones and reconfigure nucleosomes. An example of chromatin remodeling is evident in the development of an embryonic heart. Mutations or defects resulting from these remodelers can lead to a variety of cardiovascular disorders in adults.[85]

3.6. Non-coding RNA (ncRNA) expression

ncRNA refers to RNA molecules transcribed from DNA but not translated into proteins. There are four major types of ncRNA involved in regulating gene expression: MicroRNAs (miRNA), long ncRNAs (lncRNA), Piwi-interacting RNAs, and short interfering RNAs, all of which have been extensively investigated.[86] These regulatory mechanisms play pivotal roles in managing transcription and translation, influencing chromatin remodeling, and are integral to physiological processes as well as disease states.[87] MiRNAs function by binding to target RNAs, thereby suppressing gene expression and inhibiting translation. They facilitate the transfer of genetic information within cells, between different cells and tissues, and even across body fluids such as breast milk, sweat, urine, and blood. Due to their involvement in cardiovascular disorders and tumor suppression, miRNAs are considered excellent diagnostic markers.[60,64] In contrast, lncRNAs are more cell-type specific and are expressed at lower levels compared to miRNAs. They primarily regulate transcription, which may occur within or outside the nucleus. In addition, lncRNAs exhibit diverse functions, including regulation of mRNA within the cytoplasm of a cell.[86]

4. The *DRD2* gene as a therapeutic target for RDS

In a blinded study, Blum *et al.*,[88] demonstrated the initial allelic association of the *DRD2* gene with alcoholism. The study employed 70 brain samples collected from both non-alcoholics and individuals diagnosed with alcoholism. DNA samples underwent digestion with restriction endonucleases and were probed with a clone containing the complete 3' coding exon, the polyadenylation signal, and approximately 16.4 kilobases (kb) of non-coding 3' sequence of the human *DRD2* gene (lambda hD2G1). The findings revealed that the presence of the A1 allele of the *DRD2* gene accurately classified 77% of the individuals with alcoholism, while its absence correctly classified 72% of the nonalcoholics. This polymorphic pattern of the *DRD2* gene suggested the presence of a susceptibility gene for at least one form of alcoholism on the q22-q23 region of chromosome 11.

Using similar brain tissue, Noble *et al.*[23] conducted a study investigating the allelic association of the human *DRD2* gene with the binding characteristics of the *DRD2* receptor in 66 brains obtained from both non-alcoholic and alcoholic subjects. In this blinded experiment, DNA extracted from the cerebral cortex underwent treatment with the restriction endonuclease Taq and was probed with a 1.5-kb digest of a clone (XhD2G1) of the human *DRD2* gene. The binding characteristics, including the binding affinity (K_d) and the number of binding sites (B_{max}), of DRD2 were determined in the caudate nuclei of these brains using tritiated spiperone as the ligand. The results revealed that compared to nonalcoholic subjects, the adjusted K_d was significantly lower in alcoholic subjects, indicating a higher binding affinity of DRD2 in the latter group. In addition, B_{max} was found to be lower in subjects carrying the A1 allele, which exhibited a strong association with alcoholism, whereas no significant changes in B_{max} were observed in subjects carrying the A2 allele. Moreover, a progressively reduced B_{max} was found in subjects with A2/A2, A1/A2, and A1/A1 alleles, with subjects carrying A2/A2 alleles exhibiting the highest mean values and those with A1/A1 alleles exhibiting the lowest. The observed polymorphic pattern of the *DRD2* gene and its variation in receptor expression strongly suggests the involvement of the dopaminergic system in predisposing individuals to at least one subtype of severe alcoholism. Subsequent studies have corroborated these findings,[89-104] and as of February 22, 2024, a PubMed search using the term "Dopamine D2 Receptor Gene" yielded a total of 5485 listings.

While we are cognizant of epigenetic insults affecting at least seven major neurotransmitter pathways, including cannabinoidergic, cholinergic, serotonergic, opioidergic, gluconergic, GABAergic, and glutaminergic, our focus lies on the dopaminergic system, specifically the *DRD2* gene. The primary review, which is based on PUBMED listings using the search term "Methylation and the Dopamine D2 Receptor Gene," yielded 378 listings as of February 22, 2024.

5. Epigenetic modifications and *DRD2* gene

While we recognize that many reward genes and associated loci have been shown to undergo epigenetic modifications after the consumption of drugs of abuse (i.e., alcohol), the purpose of this commentary is to present a narrative that focuses on identifying epigenetic modifications, specifically in the *DRD2* gene. In this commentary, we chose not to denote DNA and/or histone methylation, but instead, we provide a summary based on behavioral effects.

A recent study by Bohnsack *et al.* provides an example of targeting and editing epigenetic histone modifications to attenuate unwanted behaviors.[104] Adolescent binge drinking is widely recognized to induce epigenetic modifications at the enhancer region of the activity-regulated cytoskeleton-associated protein (ARC) immediate-early gene, specifically known as the synaptic activity response element (SARE).[104] These epigenetic modifications lead to a decrease in *ARC* expression in the amygdala, a phenomenon observed in both rodent models and human studies.[104]

In an experiment conducted by Pandey's group, it was demonstrated that the use of dCas9-P300 resulted in increased histone acetylation at the *ARC* SARE. This intervention effectively normalized deficits in *ARC* expression and consequently led to a reduction in adult anxiety and excessive alcohol consumption in a rat model that examined alcohol exposure during adolescence.[104] Interestingly, dCas9-Kruppel-associated box (KRAB), in contrast, was found to promote repressive histone methylation at the *ARC* SARE, resulting in decreased *ARC* expression, the manifestation of anxiety-like behaviors, and increased alcohol consumption in the control group.

5.1. SUD

A summary of studies focusing on the epigenetics SUD is presented in Table 1. Detailed descriptions of SUD are provided in the following subsections.

5.1.1. Cannabis

In a study conducted by Oyaci *et al.*,[105] DNA methylation levels at the *DRD2* gene were compared among patients based on clinical parameters and *DRD2* genotype distribution. Their findings revealed significant differences in methylation status between groups, particularly concerning the presence of a family history of CUD or synthetic cannabinoid use disorder (SCUD). One notable limitation of the study is the utilization of inaccurate controls (the authors did not screen the controls for other potentially addictive behaviors such as gambling and overeating). It is known that without such screening, the presence of the *DRD2 A1* allele, for example, could be as high as 40%. This flaw could have prevented the investigators from establishing direct evidence for high DNA methylation with both CUD and SCUD. In a separate work conducted by Gerra *et al.*,[106] a genetic and sociodemographic analysis involving both CUD patients and control groups was performed to explore DNA methylation in the *DRD2-ANKKI* gene region. They identified significant hypermethylation at exon 8 of the *DRD2* gene. Interestingly, the study also uncovered a correlation between higher education levels and a reduced risk of CUD. The authors astutely proposed that their findings of differentially methylated regions (DMRs) at exon 8 of the *DRD2* gene could serve as

Table 1. Epigenetics of substance use disorder

Type of substance	Findings	References
Cannabis	Higher histone methylation onto *DRD2* gene with family history	Oyaci *et al.*[105]
	DNA methylation on *DRD2* promotor	Gerra *et al.*[106]
	Prenatal Δ-9- tetrahydrocannabinol (THC) exposure with an increase in histone methylation *DRD2* gene	DiNieri *et al.*[107]
Tobacco	DNA methylation on *NCAM1-TTC12-ANKK1-DRD2* gene cluster associated with risk for cigarettes per day	Liu *et al.*[109]
Alcohol	Enhanced global histone methylation	Pandey *et al.*[111]
	DRD2 promoter DNA methylation was positively associated with responses to alcohol severity	Bidwell *et al.*[112]
	DRD2 DNA methylation was significantly associated with alcohol problem severity	Hagerty *et al.*[114]
	DNA methylation at the *DRD2* gene was significantly increased in association with familial high-risk status	Hill and Sharma[116]
	Craving was significantly associated with *DRD2*-gene methylation	Hillemacher *et al.*[117]
Psychostimulants	METH-treated mice also showed (i) decreased global levels of total H3ac and H4ac and increased global levels of 5-methylcytosine (5-mC) and (ii) decreased H3ac enrichment at promoters of *DRD2*	González *et al.*[121]
	In both human and animal self-administration experiments, the histone mark protein-R- methyltransferase-6 (PRMT6) and asymmetric demethylation of R2 on histone H3 (H3R2me2a) decreased in the rodent and cocaine-dependent human NAc.	Damez-Werno *et al.*[122]
	METH induced a decrease in DNA methylation of a number of dopamine-related genes (i.e., *DRD3*, *DRD4*, and *COMT*) in patients with METH psychosis but not in non-METH psychosis patients.	Nohesara *et al.*[124]
Opioids	Specifically, rs4867798-CpG_174872884 and rs5326-CpG_174872884 in the *DRD1* gene showed hypermethylation	Zhang *et al.*[125]

potential biomarkers or targets for the development of pharmacological therapeutic agents.

In a study by DiNieri *et al.*,[107] utilizing an animal model of prenatal exposure to delta-9-tetrahydrocannabinol (THC), it was observed that prenatal cannabis exposure led to a reduction in *DRD2* messenger RNA expression in the NAc, a crucial brain reward region. Furthermore, Hurd's group[107] identified histone methylation differences in the offspring of pregnant rats exposed to THC. Chromatin immunoprecipitation of the adult NAc revealed an increase in the 2meH3K9 repressive mark and a decrease in 3meH3K4 and RNA polymerase II at the *DRD2* gene locus in the THC-exposed offspring. This diminished *DRD2* expression correlates with reduced *DRD2* binding sites and heightened sensitivity to opiate reward in adulthood. Moreover, the study suggested that maternal cannabis use alters the epigenetic mechanisms governing histone lysine methylation, thereby influencing the developmental regulation of mesolimbic *DRD2* in offspring. This subsequent reduction in *DRD2* may increase the susceptibility of the offspring to addiction in the future. Of interest, earlier work from Blum's laboratory[108] demonstrated significant alteration of the vas deferens from prenatal exposure to THC in that there was an increased sensitivity to enkephalins, in agreement with the findings of DiNieri *et al.*[107]

5.1.2. Tobacco

As highlighted by Liu *et al.*,[109] previous research has underscored the importance of the *NCAM1-TTC12-ANKK1-DRD2* gene cluster in addiction susceptibility among individuals of European and African descent. Building on this foundation, Liu and colleagues conducted next-generation bisulfite sequencing to uncover smoking-associated DMRs. Through haplotype-based association analysis, they identified a significant association between cigarettes per day and the C-T-A-G haplotype in *DRD2*, formed by rs4245148, rs4581480, rs4648317, and rs11214613. Furthermore, the study identified four significant smoking-associated DMRs, with three residing in the *DRD2/ANKK1* region. These findings elucidate a notable association of variants and haplotypes within the *ANKK1/DRD2* region with nicotine dependence among Chinese male smokers. Importantly, the results also underscore the pivotal role of DNA methylation in facilitating such associations.

5.1.3. Alcohol

Strong correlations have been observed between DRD2 binding potential and neural responses to rewarding stimuli and substance use.[89-104] Consequently, disruptions in DRD2 function constitute a critical component of theoretical models elucidating the pathophysiology of substance dependence.[110] Moreover, as proposed by Pandey *et al.*,[111] epigenetic modifications may serve as fundamental molecular mechanisms influencing how alcohol exposure impacts the brain.

This idea has been investigated by Bidwell *et al.*, who demonstrated that, after controlling for age, *DRD2* promoter DNA methylation was positively associated

with responses to alcohol cues in the left caudate, right caudate, left putamen, right putamen, and right Nac.[77,112] This finding indicated that robust striatal activation, in response to reward cues, was linked with DNA methylation at the *DRD2* gene. In addition, *DRD2* methylation was linked to alcohol use disorder (AUD) severity. Specifically, *DRD2* methylation was linked to scores on the AUDs Identification Test, Impaired Control Scale, and Alcohol Dependence Scale.

Chronic consumption of alcohol and various other illicit drugs has been associated with adverse consequences, including functional connectivity deficits within neural networks linked to executive control.[113] These deficits may indirectly contribute to the development of aberrant alcohol-seeking behavior.[113] Hagerty *et al.*[114] found that, specifically, average DNA methylation at the *DRD2* gene was negatively correlated with left and right executive control network connectivity, but no correlation was found with the other networks that were tested. In addition, *DRD2* DNA methylation was found to be linked with the severity of alcohol problems. These findings bolster a theoretical framework linking the neurobiological markers of alcohol consumption among polysubstance users to epigenetic influences.

Individuals with AUD tend to have dopaminergic alteration, and those with a family history of AUD may experience influences on brain development.[115] Hill and Sharma discovered a significant positive correlation between familial high-risk status and DNA methylation at the *DRD2* gene.[116] In fact, significant differences in the volume of the left inferior temporal, left fusiform, and left insula regions were observed between high-risk and low-risk familial risk groups. Previously, these regions have been associated with social cognition. In addition, the DNA methylation of the *DRD2* gene was inversely correlated with the volumes of grey matter in these regions.

Along similar lines of thinking, Hillemacher *et al.*,[117] concerned about the role of epigenetics on dopaminergic neurotransmission and its influence on alcohol dependence, found a significant increase of DNA methylation at the *DRD2* gene during alcohol withdrawal/early abstinence. Furthermore, they discovered a significant association between craving, as measured by the obsessive-compulsive drinking scale, and the DNA methylation of the *DRD2* gene. Their findings regarding this association with alcohol craving underscore the pivotal role of *DRD2* gene DNA methylation in the neurobiology of addictive behavior. It is worth noting that the inability to demonstrate significant alterations in DNA methylation between controls and patients may stem from an inadequate screening of the control group.[118]

Moreover, as previously pointed out by several researchers,[89-104] reduced ligand binding of DRD2 has consistently been observed in the striatum of individuals with AUD and other RDS behaviors. The reduced DRD2 binding has been suggested to indicate diminished DRD2 density, which, in turn, has been proposed to trigger cravings and increase the likelihood of relapse. Accordingly, Feltmann *et al.*,[119] surprisingly, unlike others, did not find DNA methylation differences in the investigated regions of the *DRD2* gene. However, in Wistar rats, chronic alcohol drinking significantly decreased mRNA levels of the long isoform of the *DRD2* gene in the NAc. In addition, alcohol drinking also decreased the striatal density of DRD2-DRD2 homoreceptor complexes, increased the density of A2AR-DRD2 heteroreceptor complexes in the NAc shell and the dorsal striatum, and decreased the density of sigma1R- DRD2 heteroreceptor complexes in the dorsal striatum. Interestingly, the chronic alcohol consumption in these rats appears to fit well with earlier findings from Blum's laboratory involving Golden Syrian hamsters.[120] In particular, compared to the control hamsters who only drank water, the experimental hamsters who freely drank ethanol after a year had a noticeably lower concentration of a leucine-enkephalin-like immunoreactive substance in their basal ganglia.[120]

This discovery suggests that ethanol's effects involve the synthesis of endogenous peptidyl opiates, which appears akin to the findings of long-term drinking, as presented in the Feltmann *et al.* study. Thus, although we cannot rule out the possibility of DNA methylation differences occurring in other regions and/or other epigenetic modifications not studied in the Feltmann *et al.*[119] study, it begs the question as to the possibility that mRNA expression suppression by alcohol drinking also reduces the synthesis of DRD2. Feltmann *et al.*[119] also provided support for the hypothesis that AUD was associated with a hypodopaminergic system and proposed the A2AR-DRD2 heteroreceptor complex as a promising novel target for treatment.

5.1.4. Psychostimulant abuse

Recently, Blum *et al.*[30] proposed that mild D2 receptor stimulation achieved by certain therapeutic modalities can cause dopamine release, which, in turn, can change D2-directed mRNA and improve DRD2 function in humans. This increase in DRD2 activity is thought to reduce craving behaviors, particularly in high-risk, genetically compromised populations. Accordingly, Cadet's group has conducted eloquent experiments comparing methamphetamine (METH) and modafinil in terms of epigenetic insults.[121]

González *et al.*,[121] demonstrated that repeated administration of either METH or modafinil to mice resulted in cognitive effects. In the medial prefrontal cortex, there

were observed inductions in histone acetylation and DNA methylation profiles. Mice subjected to repeated METH exposure, but not modafinil, exhibited impaired cognitive memory, as revealed by the novel object recognition test, with a notable decrease in memory recognition. In addition, METH-treated mice demonstrated (i) decreased levels of histone H3 and H4 acetylation and increased levels of 5-mC and (ii) reduced histone H3 acetylation enrichment at promoters of the *DRD2* gene. These findings suggest that epigenetic dysregulation, particularly at the *DRD2* gene, is associated with the long-term cognitive decline effects of METH and its adverse impacts on medial prefrontal cortex function. Further studies are warranted to elucidate the specific mechanisms of epigenetic regulation affected by psychostimulant abuse.

Changes in histone methylation and acetylation on DNA within the NAc and lysine residues have been observed with repeated cocaine administration. Nestler's group investigated histone arginine (R) methylation in models related to reward processing. Specifically, Damez-Werno *et al.*[122] found that in both animal and human self-administration experiments, the histone mark protein-R- methyltransferase-6 (PRMT6) and asymmetric demethylation of R2 on histone H3 (H3R2me2a) were reduced in the rodent and cocaine-dependent human NAc. In fact, while PRMT6 overexpression in medium spiny neurons (MSNs) expressing DRD1 (D1-MSNs) is protective against cocaine-seeking behaviors, PRMT6 overexpression in D2-MSNs in all NAc neurons has been associated with increased cocaine-seeking behaviors. Along these lines, Blum *et al.* hypothesized that dopaminylation (H3R2me2a binding) occurs in PUD, and the binding inhibitor Srcin1, like the major *DRD2 A2* allelic polymorphism, protects against psychostimulant-seeking behavior by normalizing NAc dopamine expression. Moreover, numerous studies have verified the association between the *DRD2 Taq A1* allele (30 – 40 lower DRD2 numbers) and severe cocaine dependence. According to Lepack *et al.*,[123] acute cocaine increases dopamine in NAc synapses and causes histone H3 glutamine 5 dopaminylation and subsequent inhibition of *DRD2* expression. With prolonged cocaine use, the inhibition of *DRD2* expression increases and accompanies cocaine withdrawal. Furthermore, they have reported that during cocaine withdrawal, the Src kinase signaling inhibitor 1 (Srcin1 or p140CAP) decreased H3R2me2a binding. Thus, this inhibited dopaminylation induced a "homeostatic brake." Blum's group[37] suggested that the reduction in Src signaling in NAc D2-MSNs, like the *DRD2 Taq A2* allele, a well-known genetic mechanism protective against SUD, normalizes the NAc dopamine expression and decreases cocaine cravings and cocaine-seeking behaviors. Therefore, Srcin1 could be an important target for therapeutic interventions.

Another important facet related to METH-induced psychosis may involve both genetic DNA antecedents and epigenetic post-translational changes in mRNA expression, for example, on the *DRD2* gene. Nohesara *et al.*[124] found that METH induces a decrease in DNA methylation of a number of dopamine-related genes (i.e., *DRD3*, *DRD4*, and *COMT*) in patients with METH psychosis but not in non-METH psychosis patients. The suggestion here is that, in general, METH dependency is linked with a reduction in DNA methylation and an open chromatin conformation, leading to increased expression of several important genes involved in the pathogenesis of psychotic disorders.[121] Nohesara *et al.*[124] suggested that these epigenetic modifications may serve as valuable diagnostic biomarkers for diagnosing psychosis in METH abusers. In addition, they support the use of a methyl-rich diet for the suppression or prevention of psychosis in these patients, thus encouraging further association and interventional studies involving larger populations.

5.1.5. Opioid abuse

Opioid use disorder (OUD) and other reward-dysregulated disorders have a high degree of heritability. Zhang *et al.*[125] demonstrated that various methylation quantitative trait loci (mQTLs) in the *DRD1* and *DRD2* genes were identified in both the healthy control and heroin use disorder groups. Specifically, rs4867798-CpG_174872884 and rs5326-CpG_174872884 in the *DRD1* gene were the unique single-nucleotide polymorphism-CpG pairs observed in patients suffering from heroin use disorder. This groundbreaking research suggests that certain dopaminergic mQTLs may be linked with characteristics of OUD by implicating DNA methylation and gene expression. However, it underscores the necessity for improved screening of controls to reassess the findings regarding DRD2.[120]

5.1.6. Eating disorders

Similar to SUD, anorexia nervosa is a multifaceted and highly heritable disease.[126-128] Rask-Andersen *et al.*[128] reviewed the relevant literature and discovered that 175 association studies had been conducted on an anorexia nervosa cohort, examining 128 different polymorphisms related to 43 genes. The strongest correlations indicate that certain dopaminergic genes play a key role in regulating body mass index.[129] Moreover, findings by Frieling *et al.*[130] revealed an increase in the expression of dopamine transporter (*DAT*) mRNA and a decrease in *DRD2* expression. Frieling *et al.* suggested that the upregulation of the *DAT* gene was accompanied by a hypermethylation of the gene's promoter in the anorexia nervosa and bulimia nervosa group, while significant hypermethylation of the *DRD2* promoter was only present in the anorexia nervosa

group. No discrepancies in methylation or expression were detected in the various other dopamine receptors that were examined. In addition, Groleau *et al.*[131] found that women diagnosed with bulimia spectrum disorder, particularly those with borderline personality disorder, demonstrated significant elevations in *DRD2* DNA methylation levels than women with no eating disorders. Moreover, women with a history of bulimia spectrum disorder and childhood sexual abuse showed increased *DRD2* DNA methylation compared to the group with no eating disorder history. These results suggest that individuals with bulimia spectrum disorder experience an increase in DNA methylation of the *DRD2* gene promoter, which may serve as a stronger marker of comorbid psychopathology rather than being a board correlate of eating disorders in general.

5.1.7. Gambling

According to several studies, dopaminergic circuits may play a role in the pathophysiology of problematic gambling behavior.[132-134] Hillemacher *et al.*[134] reported that DNA methylation patterns in the *DRD2*-gene were modified with respect to abstinence over a 12-month or a 30-month period. In addition, they observed increased levels of DNA methylation in individuals who were non-abstinent and those who did not seek treatment. These findings suggest that altered *DRD2* expression, resulting from variations in DNA methylation, holds pathophysiological significance in lifetime pathologic gambling behavior.

5.1.8. Personality disorders

Utilizing chimpanzees, which are known to exhibit five dimensions of personality (agreeableness, dominance, extraversion, openness, and reactivity/undependability), Staes *et al.*[135] observed that *DRD2* DNA methylation is most strongly linked to extraversion. In addition, they found that varying DNA methylation levels at specific *DRD2* sites were linked to changes in extraversion in nursery-reared, but not mother-reared, individuals. These findings provide further support for the importance of biological mother-related rearing in early life. This work further suggests that early-life experiences can influence long-lasting behavioral effects, theoretically through epigenomic modification. Accordingly, these results contribute to the increasing body of research demonstrating the critical role of the experience-dependent methylome in the development of personality.[134,136-140]

5.1.9. Maternal deprivation (MD)

Gondre-Lewis's group correctly points out that stress experienced early in life can trigger a complex neurochemical cascade, affecting the emotional and addictive behaviors of adolescents and adults later in life.[141] Moreover, research indicates that drug-seeking behavior and excessive alcohol drinking are often comorbid with depressive-like symptoms and behaviors.[142] These behaviors are frequently observed in individuals who faced adversity in their early life and are important features found in animal models of early life stress exposure.[143] In fact, Guo *et al.*[144] reported that, in rats, the rats subjected to MD, chronic unpredictable stress (CUS), and a combination of both (MD/CUS) displayed increased levels of *DRD2* promoter DNA methylation compared to normal controls. The authors concluded that early-life MD increased vulnerability to stress-induced depressive-like behavior in adult rats. In line with the findings of Gondre-Lewis's group,[141-143] increased methylation of the *DRD2* promoter gene in the ventral tegmental area may raise the risk of depression and alcohol-seeking behavior. Furthermore, Zhu *et al.*[145] explored the effects of MD on adult rats' spatial learning and memory, exploratory, and limbic activity, along with their association with the expression of *DAT*, *DRD1*, *DRD2*, and *DRD3* in the NAc. In addition, Zhu *et al.*[145] investigated the potential involvement of DNA methylation in the regulation of *DRD2* gene expression. Based on their work, only the *DRD2* mRNA level was associated with total distance. However, the expression of DNMT1 and 3 alpha (DNMT3A) and the methylated CpG levels in the promoter region of the *DRD2* gene were not significantly altered in the MD group compared to the control group.[120] Functional differences may have occurred in other sites at the *DRD2* genes not included in the study, and/or other epigenetic modifications were associated with *DRD2* mRNA expression. Notwithstanding, it is possible that deficient control screening and interpretation may have impacted the association study, and further research with carefully selected controls, not just MD, may alter these results.

5.1.10. Social defeat stress

In terms of social defeat stress, Zhu *et al.* reported that chronic social defeat stress regulates FOSB expression in the NAc, which promotes the cell-type-specific accumulation of ΔFosB in the two MSN subtypes in this region. ΔFosB is selectively induced in D1-MSNs in the NAc of resilient mice and in D2-MSNs of susceptible mice.[146] In this regard, Hamilton *et al.* reported that FOSB-targeted histone acetylation in D2-MSNs or histone methylation in D1-MSNs promotes a stress-susceptible, depressive-like phenotype, while histone methylation in D2-MSNs or histone acetylation in D1-MSNs enhance resilience to social stress as measured by social interaction behavior and sucrose preference.[147] Importantly, the study presented the first evidence of targeting histone modifications specifically to genes and cells, simulating real-world transcriptional processes that regulate social defeat stress behavior.

6. Effector moieties

The effector moieties are molecules that selectively bind to proteins to modify their biological activities. These molecules, essentially ligands, can modify enzyme activity, genomic activity, cell signaling, and other protein functions.[35] While identifying epigenetic-induced post-transcriptional methylation or acetylation on chromosome histones is crucial, one of the first and most successful effector moieties in editing transcriptional activator domains was VP64, which is derived from the herpes simplex virus. VP64 recruits RNA polymerase ll directly for gene-targeted transcriptional activation.[148] It is now understood that by targeting VP64 to the promoter region of a single gene using DNA-binding domains such as ZFP,[149] TALE,[150,151] or CRISPR/dCas9,[152] laboratories can consistently induce transcription of targeted genes *in vitro* and even *in vivo*.[153,154]

Furthermore, to attain bidirectional control over gene expression, the suppression of endogenous genes is typically achieved using KRAB effector moieties. KRAB represents a transcriptional repression domain present in human zinc finger transcription factors. It functions by recruiting heterochromatin-forming complexes that subsequently deposit H3K9me3 repressive marks, resulting in transcriptional suppression.[155] Similar to VP64, the KRAB domain modifies the epigenome by enlisting the secondary factors rather than utilizing enzymatic activity. In addition, promoter- or enhancer-targeted KRAB has been used in cell culture and in the brain.[156,157] In addition, research has identified specific transcription factors and epigenetic readers, erasers, and writers associated with the addiction pathogenesis. More recently, a number of effector moieties have been developed for neuroepigenetic editing. These effector moieties include DNA-modifying enzymes such as DNMT3A (responsible for CpG methylation).[158,159] and TET1 (which catalyzes hydroxymethylation of CpG).[160,161] They also encompass proteins involved in regulating histone PTMs such as NFκB subunit p65 (which recruits HATs to acetylate core histones).[162] histone methyltransferase G9a (which catalyzes H3K9me2),[162,163] p300 HAT (which acetylates all four core histones),[164] Sin3-interaction domain (which recruits HDACs),[165,166] lysine-specific demethylase 1 (which demethylates H3K4 and H3K9),[167] PRDM9 (which methylates H3K4 and H3K36),[168] and DOT1L (which methylates H3K79).[169,170] Furthermore, transcription factors such as CREB have also been utilized, thus mimicking known mechanisms of transcriptional regulation.[171]

7. Research outlook in epigenetics of SUD

The evidence presented in this commentary strongly indicates the significant role of epigenetic (mis) regulation in SUD. Numerous associations and interventional studies demonstrate that epigenetic mechanisms are responsible for the complex regulation of dopamine homeostasis, particularly concerning the expression of the *DRD2* gene. Epigenetic regulatory mechanisms (i.e., histone modifications, ncRNA, and DNA methylation) interact in an orchestrated fashion to establish phenotypes influenced by genetic background (e.g., the presence of mutations) and environmental factors (e.g., the presence of active compounds from drugs, alcohol, and tobacco). Importantly, these interactions occur in a tissue- and cell-specific manner. Hence, a detailed mapping of epigenetic modifications across various presentations of SUD will enable the understanding of the precise regulatory mechanisms involved in each case and identify potential targets for therapeutic interventions.

The utilization of effector moieties emerges as a promising intervention. However, caution is warranted due to the pleiotropic effects of epigenetic modifiers (e.g., DNMTs, HDACs, and HTAs), and systemic inactivation of these enzymes may lead to undesirable side effects, hindering their clinical translation. Further research is needed to elucidate mechanisms for cell-specific activation or delivery of the effector moieties to mitigate side effects. Moreover, given that prenatal SUD may impact offspring health, mechanistic and interventional studies are imperative to determine and target epigenetic modifications, aiming to mitigate the effects of prenatal SUD on fetal health.

8. Summary

SUD often entails persistent behavioral abnormalities observed in susceptible individuals following repeated exposure to psychoactive drugs of abuse. The enduring nature of these behavioral alterations suggests potential long-lasting changes in gene expression within specific brain regions, which may contribute to the addiction phenotype. Advanced research over the past decade has revealed the pivotal involvement of epigenetic mechanisms in orchestrating lasting alterations, whether positive or negative, in gene expression across various tissues, particularly in the brain. This understanding has spurred investigations aimed at elucidating the impact of epigenetic regulatory processes in mediating the enduring effects of psychoactive substances of abuse on the brain, predominantly using animal models of drug addiction. However, more recently, human neuroepigenetic research has been rapidly emerging. Compelling evidence indicates that repeated exposure to drugs of abuse induces alterations within the brain's reward regions through three primary modes of epigenetic regulation: Histone modifications such as acetylation and methylation, DNA methylation, and ncRNAs. In this commentary, our focus lies on

investigating epigenetic modifications to the *DRD2* gene to directly illustrate the role of these epigenetic changes in addiction-related behavioral abnormalities. At present, there is a growing awareness regarding the utilization of various effector moieties to counteract these undesirable negative epigenetic changes, as recently observed by Pandey's group[104] in mitigating alcohol-induced anxiety.

Experimentally, effector moieties are providing unprecedented information relevant to not only mechanistic insights but also to demonstrate the importance of removing these negative epigenetic insults, leading to the concomitant attenuation of specific mRNA transcriptional expression, such as the *DRD2* gene. One important unanswered question is whether induction, for example, of dopamine homeostasis, would increase the conversion of post-transcriptional histone methylation to acetylation. Our laboratory has been developing a nutraceutical complex to gently induce pro-dopamine regulation, potentially in both animal models and humans. To answer this profoundly important question, we advocate for the scientific community to perform the necessary research in this arena.

9. Conclusion

The *DRD2* gene has been extensively investigated in various neuropsychiatric disorders. Numerous international studies have been performed since the initial association of the *DRD2 Taq A1* allele with severe alcoholism in 1990. In our opinion, the primary cause of negative reports regarding the association of various *DRD2* gene polymorphisms is the inadequate screening of controls, failing to eliminate many hidden RDS behaviors. Moreover, pleiotropic effects of *DRD2* variants have been observed in neurophysiological, neuropsychological, stress response, social stress defeat, MD, and gambling disorder contexts, where epigenetic DNA methylation and negative histone post-translational methylation have been identified, as discussed in this commentary. As of October 19, 2022, there are 70 articles focusing on DNA methylation and 20 articles on histone methylation are listed in PUBMED. Importantly, Blum and Noble characterized the *DRD2 Taq A1* allele not as specific to alcoholism but as a generalized reward gene. Therefore, it now behooves the field to find ways to either use effector moieties to edit the neuroepigenetic insults or possibly harness the idea of potentially removing negative mRNA-reduced expression by inducing dopamine homeostasis. This represents a futuristic laudable goal.

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Conflict of interest

Kenneth Blum holds patents, both domestic and foreign, related to pro-dopamine regulation complexes and genetic testing for addiction risk. Other authors declare no conflict of interest.

Author contribution

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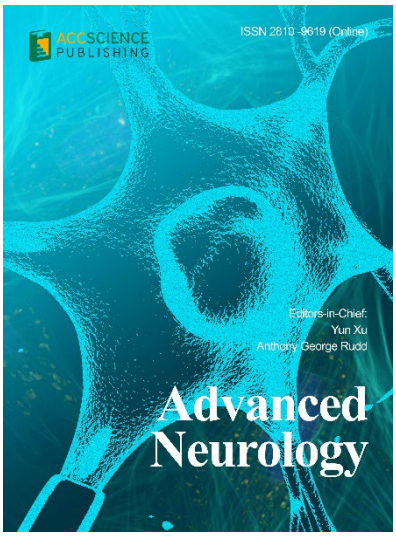

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