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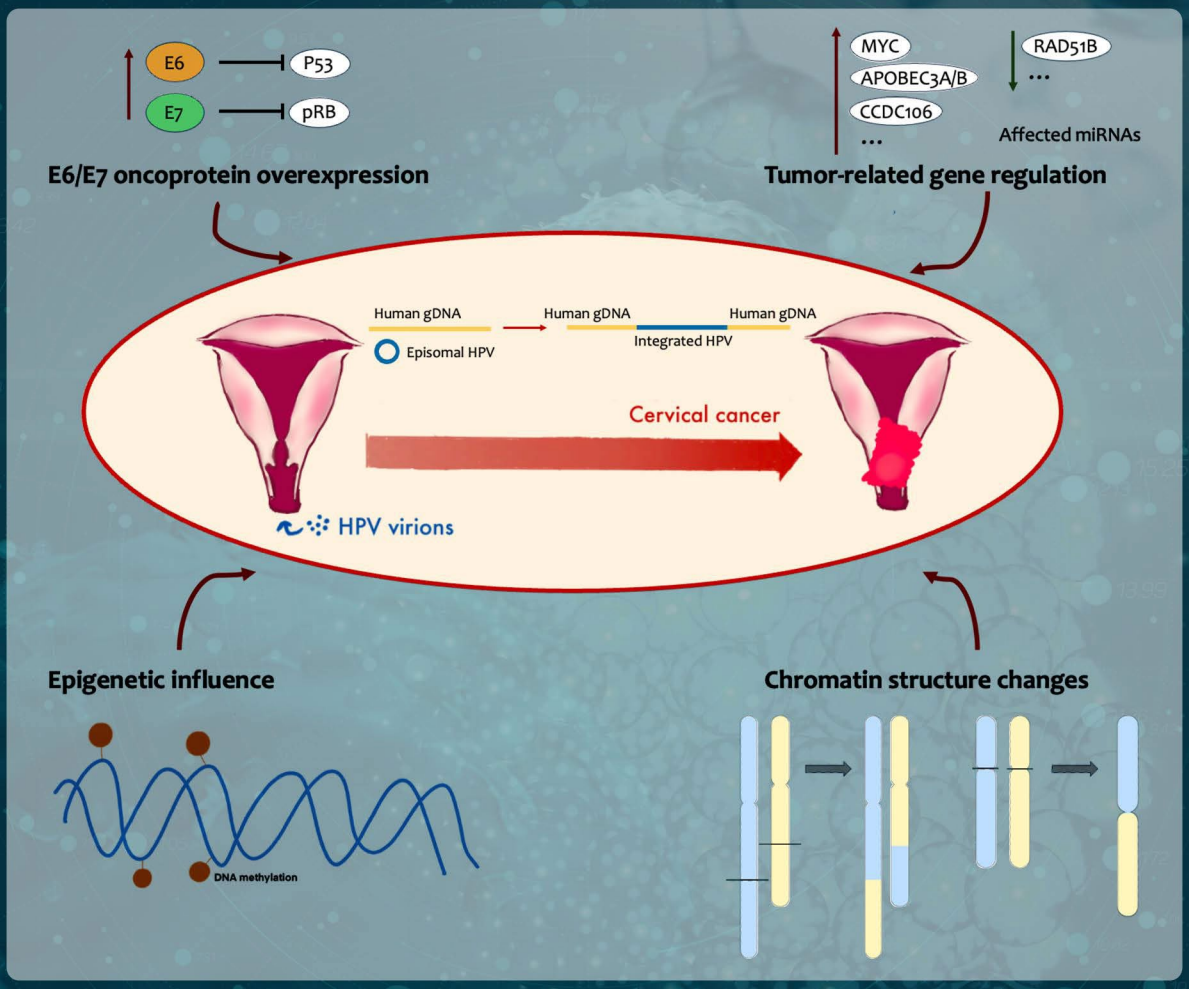

The significance of human papillomavirus integration in carcinogenesis and the development of specific diagnostics and countermeasures

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EDITORIAL

A message from the Editor-in-Chief, Prof. Lemin Zheng

Lemin Zheng†*

The Institute of Cardiovascular Sciences, Peking University, Beijing, China

The year 2023, which is the 2nd year since *Global Translational Medicine (GTM)* was launched, saw a steady growth of the journal, accompanied by several accomplishments that we are proud to share with our readers. The most encouraging achievement, unquestionably, is the inclusion of *GTM* in the CAS databases. Such indexing accomplishment is a testament to the growing scientific recognition and citation impact of the journal, paving the way for inclusion in other indexing services, including PubMed, in the future.

GTM also celebrates a surge in prominence in its field as more and more scientists are inducted to the editorial board, which plays a significant role in supporting the journal and devising plans for the strategic development of the journal. In addition, over the year 2023 we witnessed an increasing number of submissions to *GTM*, convincing evidence that the general interest in submitting to open-access journals in the field of translational medicine remains uneroded. *GTM* had a humble beginning in 2022, with a total submission count of only 38. However, this number has risen to more than 100 in 2023, accompanied by published papers which is twice the amount in 2022. Such success is not possible without the evaluative efforts of our peer reviewers from different countries, backgrounds, and expertise areas. We are grateful for the contributions of the peer reviewers who directly help enhance the academic and scientific quality of *GTM*. A more robust peer-review practice also signals that the article rejection rate will be increased so as to safeguard the journal's quality as a whole.

We believe that 2024 is going to be an eventful and remarkable year for *GTM*. In particular, we plan to hold an academic conference in the name of the journal in 2024. Thus, we are in the middle of seeking abstract submissions describing transformative works from around the world. Aside from that, *GTM* is forming academic alliances with prestigious academic and government institutions to expand our influence and invite high-quality research papers from these institutions.

We are grateful for having a strong editorial team for maintaining high academic quality for the journal as well as upholding professional standards. Our gratitude also goes to the in-house editors, who always stay committed to serving every author, for

†Professor Lemin Zheng is the Editor-in Chief of *Global Translational Medicine*

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the high-quality services they provide. Furthermore, the efforts made by every editor and reviewer have slowly but significantly strengthened *GTM* as an excellent journal and kept the journal on the right trajectory toward more shining accomplishments in the near future.

We are looking forward to pushing *GTM* to new heights next year.

Conflict of interest

The author declares no conflict of interest.

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Global Translational Medicine

REVIEW ARTICLE

Efficacy of ketogenic and low-carbohydrate diets in the management of Type 2 diabetes: A narrative review

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(This article belongs to *Special Issue: Recent Advances in Macronutrient Metabolism*)

Abstract

Obesity and diabetes represent two prevalent metabolic challenges intricately linked to poor dietary habits and a sedentary lifestyle. The escalating incidence of both conditions in recent years has approached epidemic proportions, with concomitant associations observed in individuals with excessive body weight, including hypertension and cancer. In response to this growing health concern, treatment approaches such as food therapy are deemed necessary. A pivotal aspect in managing these conditions is the careful selection of an appropriate diet to facilitate effective weight loss while minimizing potential adverse effects. Consequently, the ketogenic diet (KD) has garnered attention and support in the treatment of obesity and diabetes. This review aims to discern the potential advantages and risks associated with the utilization of a low-carbohydrate diet in Type 2 diabetic patients. It is well-established that dietary choices significantly impact the health of diabetic patients, and therefore, adopting an appropriate diet is crucial. The KD has demonstrated positive effects on blood sugar levels and glycosylated hemoglobin (HbA1c) levels, concurrently contributing to a reduction in insulin requirements during medication therapy. Furthermore, short-term experiments have revealed a positive association between nutrition choices and weight management. Beneficial improvements have been noted in the lipid profiles, including high-density lipoprotein, low-density lipoprotein, HbA1c, and triglyceride levels. For individuals grappling with diabetes or obesity, a low-carbohydrate diet emerges as a genuine and potentially beneficial therapy option. This review provides a comprehensive overview of the key concepts influencing the treatment of obesity and Type 2 diabetic patients through low-carbohydrate diets.

Keywords: Keto diet; Type 2 diabetes; Obesity; Non-communicable disease

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1. Introduction

Obesity and diabetes are increasing at an alarming rate[1]. Certain physicians and health professionals advocate for the authenticity and efficacy of low-carbohydrate diets as a therapeutic strategy for addressing diabetes and obesity[2]. The prevalence of diabetes and obesity, impacting over one billion individuals, constitutes a significant

public health hazard, potentially impairing individual work efficiency[3].

Since 1970, the global incidence of obesity has tripled, marking a substantial increase in prevalence and severity. Obesity has emerged as a primary underlying factor contributing to long-term disorders, including metabolic syndromes, vascular malformations, chronic renal disorders, malignancies, and Type 2 diabetes mellitus (T2DM), leading to a persistent rise in both mortality and morbidity rates[4]. Diabetes, recognized as a predominant cause of mortality and morbidity, particularly through the progression of cardiovascular disease, is a pressing concern in the United States[5]. The number of individuals diagnosed with diabetes exceeds 30 million, with an annual escalation in the population of overweight or obese individuals. Projections suggest that approximately 30 – 40% of the country's total population may fall into the category of overweight or obese by the year 2030, indicating a persistently high prevalence of these conditions in the United States[6].

An important global public health concern revolves around the economic burden of T2DM[7], a burden that exhibits variability across dimensions such as age, education, income, geography, race/ethnicity, and other social determinants of health. Individuals with lower levels of education and household income distinctly shoulder a greater burden than their counterparts with higher socioeconomic status, and these disparities have widened over time[8].

The International Diabetes Federation estimates that by 2040, the global diabetic population will reach 642 million, reflecting an annual escalation in disease frequency. T2DM entails both direct and indirect economic costs, with the former comprising a substantial portion of the overall economic burden. This includes direct medical resource consumption, encompassing inpatient and outpatient costs, prescription expenses, diagnostic tests, and emergency department charges[7]. In addition to the direct (healthcare-related) costs, T2DM imposes significant indirect costs, notably work productivity losses due to factors such as absenteeism or impaired working ability in the workplace attributable to illness. In addition, untimely retirement and premature deaths resulting from the disease contribute to this economic impact of reduced productivity. Importantly, these consequences extend beyond the individual, affecting family, society, and the country at large. The ramifications include implications for income, taxes, and a decline in gross domestic product[9].

Considering this issue, world leaders have committed to reducing the burden of non-communicable illnesses, including diabetes, by one-third through universal health coverage and affordable essential medicines to achieve the sustainable development goal targets for 2030[10].

Low-carbohydrate diets have immensely improved T2DM, glycemic disorders, and obesity[11]. However, the evidence supporting the efficacy of low-carbohydrate dietary approaches in treating T2DM and obesity over the long-term remains insufficient[12]. It is noteworthy that the ketogenic diet (KD) gained prominence as the principal therapeutic option for managing diabetes before the introduction of insulin in the early 20th century[12]. In 1921, Russell Wilder pioneered the use of KD to treat epilepsy. An increase in the use of KD for rapid weight loss represents a relatively recent development. This approach has demonstrated significant effectiveness within a short period of time, involving adherence to specific recommended and restricted foods (Table 1). Effective implementation of KD has been associated with weight loss, improved insulin sensitivity, and a reduction in hyperinsulinemia[13].

Dr. Elliot Joslin's diabetic diet primarily comprised fat (75%), protein (20%), and carbohydrates (<5%)[1,14]. Similarly, Dr. Allen, a medical expert, advocated for a low-carbohydrate diet for the treatment of T2DM[14].

While the necessity for appropriate dietary habits has diminished with the utilization of insulin as a restorative practice for diabetes, obesity remains intricately linked to the development of hyperglycemia, diabetes, and other related metabolic disorders. Individuals who are overweight or obese exhibit a heightened susceptibility to various diseases. Conditions such as diabetes mellitus, high cholesterol, elevated lipid levels, and disorders in energy metabolism are prevalent among this demographic[15]. Addressing obesity not only mitigates these risks but also significantly reduces the likelihood of other global health crises, including diabetes, cardiovascular diseases, and other non-communicable diseases.

Improving insulin sensitivity in T2DM is often accompanied by carbohydrate intolerance. Carbohydrate deprivation (as observed in KD) can significantly enhance blood glucose control, while weight loss can positively impact insulin resistance (IR). The potential of KD as a treatment for conditions such as obesity and T2DM has generated considerable interest. However, the available information on its efficacy remains insufficient and the associated risks to individual diets are noteworthy[16]. The correlation between obesity and diabetes has been extensively explored in numerous research. This article specifically focuses on elucidating the various mechanisms through which the low-carbohydrate diet can mitigate obesity and reduce the risk of T2DM. The aim of this narrative review is to formulate a comprehensive understanding of the role of KDs in addressing the challenges of obesity and T2DM.

Table 1. Recommended and restricted foods in the ketogenic diet[17,18]

Recommended foods	Examples	Restricted food	Examples
Protein	Fish: sardines, tuna, lobster, prawns, and shrimps	Carbohydrates	Bread, cakes, flour, honey, macaroni, noodles, potatoes, rice, spaghetti, sugar, and sweets
	Poultry: Eggs, chicken	Fruits/drinks	All kinds of fruit juices and all kinds of soft drinks
	Cheese: Full-fat cheese		
	Meat: Kabab, minced, sausages		
Oil	Flaxseed oil and olive oil mixed with salad (5 tablespoons)		
Vegetables/fruits	Avocado, artichoke, 10 berries per day, carrot, cabbage, celery, cauliflower, coriander, cucumber, eggplant, green pepper, lemon, leek, lettuce, mushroom, mint, mulberry, okra, parsley, spinach, radish, tomato, 10 – 15 olives per day, six strawberries per day, and watercress		

2. Methodology

An extensive literature review was conducted to identify pertinent original research and review papers. The search spanned various databases, including Science Direct, Google Scholar, Pub Med, Hindawi, Web of Science, and the Cochrane Library. Several keywords were employed in the literature review, encompassing "Keto diet and Type-2 diabetes," "Very low carbohydrate diet," "Low carbohydrate diet," "Ketogenic diet," "High fat diet," "Obesity and keto diet," and "Advantages and disadvantages of keto diet." Maintaining objectivity was a paramount consideration throughout this review. No specific research database, group, publisher, or country was given preferential treatment during the search and review process. Research articles were searched with unbiased titles, ensuring a balanced approach. There was a deliberate effort to neither exaggerate nor emphasize any particular aspect, whether merit or demerit, of keto diets concerning weight management. Therefore, with a considerable number of randomly selected original research articles, the potential for bias in this review is deemed minimal. In addition, this narrative review incorporated highly cited research articles on KD and T2DM from multiple reputable journals, excluding papers from predatory journals and those unrelated to KD and T2DM. Following the extensive review, 85 papers were initially selected. After eliminating duplicate papers, invited editorials, short reports, and unrelated papers, a refined set of 30 papers formed the basis for this study. Figure 1 illustrates the sorting process.

3. Classification of KD

3.1. Classical KD (CKD)

The more restrictive variant of KD is known as the CKD. In this regimen, the ratio of fat to combined protein and carbohydrate is at 4:1 by weight. In this diet, approximately 80 – 90% of total energy is derived from fats, with the remaining 10% encompassing the necessary protein percentage to meet daily requirements and the estimated carbohydrate consumption calculated from the remaining calories. This highly carbohydrate-limiting approach facilitates the rapid induction of ketosis. Individuals following CKD must curtail protein consumption to prevent the decomposition of amino acids and subsequent glucose formation. Due to its restrictive character, CKD has predominantly been employed in the treatment of epilepsy. Its application necessitates significant commitment, with patients required to undergo 50 h of dietetic counseling, incurring substantial expenses[19]. Parenteral and oral formulas designed for newborns and children are also available and can be adapted for adult use. Despite its potential benefit, CKD poses significant challenges due to its overall carbohydrate restriction, demanding a higher degree of patience and careful consideration in its implementation, which can be deemed undesirable by some individuals.

3.2. Atkins diet (AD)

Dr. Robert Atkins introduced the AD as a measure to prevent weight gain. In 1972, Dr. Atkins was the first to elucidate the principles of the AD[20]. This dietary program restricts carbohydrate intake while permitting a moderate amount of protein. The AD is divided into four distinctive phases. In the initial phase (Phase 1), carbohydrate intake is highly limited, with fewer than 20 g permitted each day. As the program progresses to Phase 2, carbohydrate intake is increased to a range of 30 – 55 g daily. During Phase 3, individuals are allowed more than 80 g of carbohydrates per day. This phase is generally repeated until the targeted body weight is achieved. In Phase 4, individuals can consume around 105 – 110 g of carbohydrates per day.

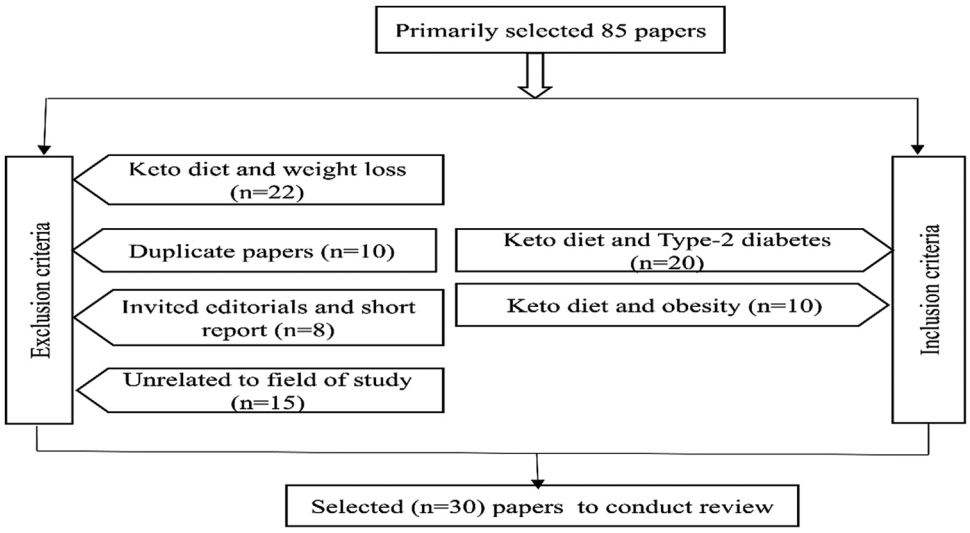

Figure 1. Articles selection process.

This cyclical approach is designed to facilitate sustained weight loss. Throughout the progression of AD, the regulatory framework becomes more flexible, allowing for the inclusion of a broader range of foods and increased carbohydrate intake. It is noteworthy that the state of ketosis, a characteristic of the initial phase, is no longer a primary focus as the individual advances through the phases.

3.3. Modified Atkins diet (MAD)

In contrast to CKD, the MAD was developed by Johns Hopkins Hospital as a less restrictive approach to treating epilepsy. Similar to AD, there is an emphasis on maintaining the initial phase for an extended duration in MAD, avoiding a transition to less restrictive stages and thereby allowing for the persistent state of ketosis. Notably, MAD, like AD, was not developed with the primary goal of weight loss. Therefore, the major restrictions pertain to low carbohydrate intake and calorie content, with no prohibitions on liquids or protein consumption. Whether adhering to a carbohydrate limit of <20 g/day or adopting a flexible supply of peptides, the predicted carbohydrate-to-protein ratio is approximately 1:1, resulting in a more palatable diet[20]. It is important to note that dietary fiber is not factored into the overall carbohydrate count, although sugar alcohols should be considered in the calculation[21].

3.4. Medium-chain triglyceride KD

In 1971, Huttenlocher developed a form of KD aimed at improving adherence among individuals with epilepsy[22]. This approach, known as the medium-chain triglyceride KD (MCTKD), relies on a sophisticated process that facilitates the utilization of moderately ketone body-bound (C7 – C13) triglycerides in comparison with long-chain triglycerides. This preference may stem from the fact that decanoic and octanoic acids, abundant, and easily digestible, are efficiently transported to the liver, where they undergo β-oxidation to produce β-hydroxybutyrate, acetoacetate, and acetone[23]. As the ketogenic potential intensifies, MCTKD demands a lower intake of saturated fats to sustain an equivalent level of ketosis as observed in CKD, enabling a reduction in the ratio of fat to combined protein and carbohydrate. Intermediate triglycerides should constitute about 65% of total calorie intake, allowing for increased carbohydrate consumption and yielding a more palatable diet. Notably, a significant complication associated with MCTKD is a higher prevalence of gastric problems, such as indigestion, diarrhea, and vomiting[20]. As a consequence, several enhancements to MCKTD have been recommended, involving an additional implementation stage characterized by an increase in the proportion of intermediate triglycerides and a dynamic balance between intermediate to long-chain triglycerides[24]. Individuals have been deemed capable of receiving 40 – 50% of their energy from medium-chain triglycerides without experiencing adverse effects[23]. Studies suggest that this diet may improve the efficacy of chemotherapy in the treatment of cancer and the prevention of recurrent cancer[25].

3.5. Low glycemic index treatment (LGIT)

The LGIT, established in 2002, represents the least restrictive form of KD[26]. Carbohydrate consumption is set at 40 – 60 g daily, with an emphasis on choosing only carbohydrates with a low glycemic index (<50)[27]. As part of the LGIT protocol, high-glycemic meals such

as rice, beer, and white bread, as well as starchy foods, are to be avoided. Instead, the focus is on incorporating low-glycemic-index foods such as leafy greens, beans, grains, lactose, and animal fat into the diet (Table 2).

4. Dietary management for Type 2 diabetes patient

The KD revolves around minimizing carbohydrate intake while emphasizing a high consumption of good, healthy fats. Simultaneously, dietary guidelines emphasize the importance of incorporating plenty of fruits and vegetables. A well-rounded diet should ideally include macronutrients such as carbohydrates, proteins, and fats. Conventionally, it has been proposed that a normal diet consists of approximately 50 – 65% carbohydrates, 10 – 15% proteins, and no more than 30% fat[1]. The American Diabetes Association has established a dietary pyramid specifically targeting diabetic patients (Figure 2)[29]. Current regulations from the Polish Diabetes Association stipulate that carbohydrates should contribute 45 – 50% of energy in consumable diets. However, European and United States regulations prescribe a broader range of 45 – 65% for these constituents in individuals with diabetes. This variation appears to be associated with potentially significant changes in blood sugar control, the maintenance of healthy body weight, and the regulation of triglyceride levels among adults with diabetes. This recommendation is particularly relevant to carbohydrates derived from high-fiber foods such as vegetables, whole grains, breads, and cereals. Individuals with T2DM frequently receive this management approach along with a low-carbohydrate diet. The term "low-carbohydrate diet" is used to refer to various dietary approaches that may vary in carbohydrate, protein, and fat consumption (Table 3).

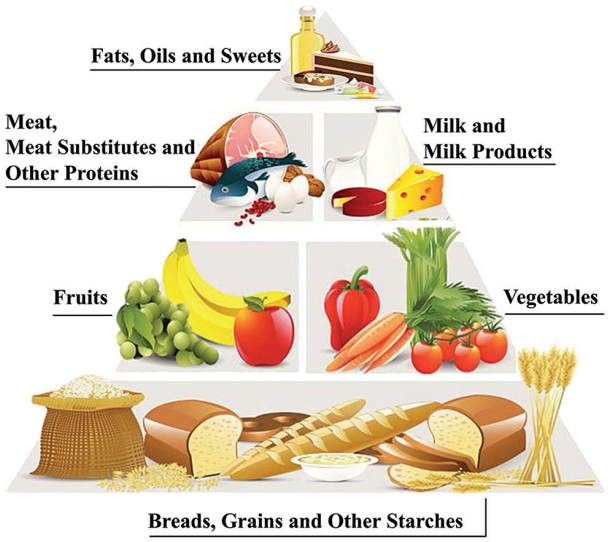

Figure 2. The American Diabetes Association's food pyramid for diabetes patients[29].

Table 2. Guideline for recommended carbohydrate intake and energy supply in ketogenic diets[28]

Description	Recommended carbohydrate intake per day (g)	Carbohydrate supply energy (%)
Ketogenic diet (very low-carbohydrate diet)	<20 – 50	<10
Low-carbohydrate diets	<130	<26
Moderate carbohydrate diets	130 – 230	26 – 45
High carbohydrate diets	>230	>45

5. Mechanisms of production and utilization of ketone bodies

The body produces ketone bodies due to a lack of sufficient carbohydrates. Historically, ketone bodies were thought to result from abnormal respiration caused by inadequate fat oxidation due to a lack of glucose intake[33]. These ketone bodies acquired labels such as "diabetic trash" or "oxidative baddies" due to this perspective. Although ketone bodies are present in circulation during both fueled and starved phases, blood concentrations are typically small under normal circumstances in humans, ranging between 0.1 and 0.4 mM.

Blood levels of ketone bodies in individuals vary, ranging from 1 mM to 5 mM after brief fasting (3 – 4 days) to 8 – 9.5 mmol after prolonged fasting (17 – 24 days)[34]. Individuals typically generate blood ketone body contents of 1 – 2 mM after workouts[35]. Plasma concentrations of ketone bodies can exceed 5 mM when individuals consume limited carbohydrates or adhere to KDs[35].

Ketone bodies are compounds featuring a carbonyl carbon, and their geometry is indicated by the formulae RC(=O)R', wherein R and R' can represent a multitude of carbon-containing methyl groups. These bodies are primarily produced within the mitochondria of the

Table 3. Nutrient levels in diverse types of diets[21,30]

Diet	Macronutrients		
	Carbohydrate (%)	Protein (%)	Fat (%)
Atkins diet*	3 – 16	28 – 34	55 – 65
Kwasniewski diet	9.2	14	76.8
Zone diet	40	30	30
Guidelines for balanced nutrition[31,32]	55 – 65	10 – 15	25 – 35

Note: *This proportion range is calculated based on individuals' scenarios.

hepatocellular system, serving as an alternate fuel source for most organs. The systemic contribution of ketone bodies to fuel ranges from 2% to 6% during prolonged fasting, escalating to approximately 30 – 40% after 2 days of fasting[36]. The three key ketone bodies synthesized from hepatocytes are acetoacetate, acetone, and 3-β-hydroxybutyrate. The latter is theoretically classified as a β-hydroxy acid rather than a true ketone body. Among these, 3-β-hydroxybutyrate appears to be the predominant ketone body molecule in plasma, followed by acetoacetate. Notably, volatile acetone is predominantly lost through respiration and is typically undetectable in the blood.

The synthesis and subsequent decomposition of ketone bodies, referred to as ketogenesis and ketolysis (Figure 3), are regulated by glucose and insulin secretion, respectively, effectively managing plasma concentrations of ketone bodies. The ketogenesis reaction is catalyzed by glucagon, while the production of ketone bodies is decreased by insulin[37]. This typical post-absorptive fluid ketogenic value in a healthy adult is approximately 0.12 mM/L; however, obese adults exhibit a higher ratio (0.42 mM/L)[38]. In diabetic patients experiencing low blood sugar, ketone body levels exceed 26.5 mM/L. It is also projected that achieving blood ketone body levels within the range of 8 – 9.5 mM/L is necessary to elicit the protective impact associated with a KD[39].

Ketone bodies are four-carbon short chains accommodated in organ mitochondria through a process termed "ketogenesis"[40]. This process initiates as fatty acids as lipolyzed into fatty acids, subsequently transferred from white adipose tissue to hepatic cells, and transformed into acetyl coenzyme A (CoA)[37]. Acetyl CoA aggregates in a moderate environment and is then metabolized to acetoacetyl CoA by the enzyme thiolase. Hydroxy-methylglutaryl (HMG) CoA oxidase catalyzes the biosynthesis of 3-hydroxy-methylglutaryl CoA, leading to the conversion of HMG CoA to acetoacetyl CoA and acetoacetate. Acetoacetate can be hydrolyzed to 3-hydroxybutyrate through 3-β-hydroxybutyrate hydrogenase or converted to acetone without enzyme involvement. Unused acetone, no longer in use, is nevertheless primarily excreted through urination and released through respiration.

Acetoacetate and 3-β-hydroxybutyrate both circulate and reach non-hepatic tissues almost simultaneously. Ketosis becomes necessary to utilize energy derived from ketone bodies. Following ketolysis, succinyl CoA:3-oxoacid CoA transferase (SCOT) and acetyl CoA acetyltransferase metabolize acetoacetate and 3-hydroxybutyrate, converting them back to acetyl CoA (ACAT1). Acetyl CoA then enters the Krebs cycle, where it undergoes complete combustion, yielding 22 ATP per molecule[37]. Although the liver is the main source of ketone bodies, its capacity to metabolize them is hindered by the absence of SCOT. Figure 3 illustrates this simplified depiction of ketogenesis and ketolysis.

6. Diabetes mellitus

Diabetes is a chronic disorder resulting from insufficient insulin production or hyperinsulinemia, associated with persistent hyperglycemia and impairments in carbohydrate, lipid, and protein metabolism[41]. Absolute

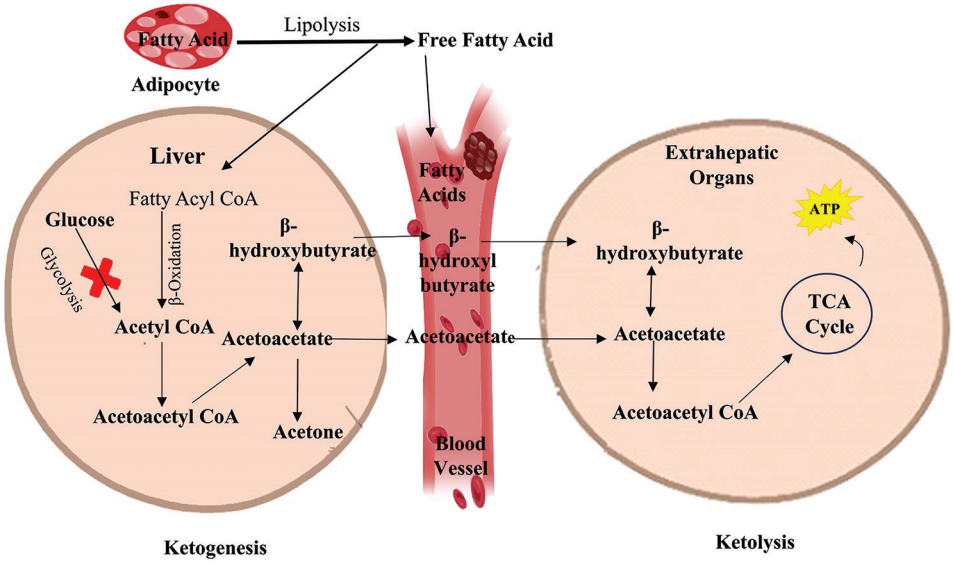

Figure 3. Production of ketone bodies (ketogenesis) and utilization of ketone bodies (ketolysis).
Abbreviations: ATP: Adenosine triphosphate; CoA: Coenzyme A; TCA: Tricarboxylic acid cycle.

insulin deficiency occurs only when the pancreas is unable to produce appropriate insulin, or the supplied insulin is not adequately used by the body's tissues and organs. There are three main types of diabetes mellitus[42].

Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), and gestational diabetes are among the diverse subtypes of diabetes. Individuals with T1DM are not generally obese, yet they still have a chance to develop complications of diabetic ketoacidosis[43]. A body mass index (BMI) greater than 30 kg/m^2 is an important point in T2DM[44] and is correlated with the development of IR in physiological problems[44]. Although obesity appears to promote T2DM and IR, the specific mechanism is unexplained. The physiology of this disorder is characterized by a mechanical failure in the feedback effects in between the proper functioning of insulin or insulin ejection, resulting in fatigue and exhaustion in hyperglycemia[45]. Metabolic derangement promotes T2DM by promoting both β-cell destruction and IR[46]. The pathogenesis of β-cells may be associated with cell apoptosis[47].

Being overweight, leading to increased blood sugar and high cholesterol, consequently fosters long-term inflammation in the body and IR. The cytoplasmic membrane, inflammation, the respiratory tract, and amyloid stress are among the perilous conditions to which cells are exposed as a result of differences in their responsiveness to genomes. This leads pancreatic cells to lose their adhesion[48]. Subsequently, this triggers suicidal unfolded protein response (UPR) systems, resulting in an oversupply of free fatty acids (FFAs), hyperglycemia, and cell malfunctioning by amplifying endoplasmic reticulum (ER) stress[49]. In addition, obesity-related clinical disorders, lipotoxicity, and glucolipotoxicity induce reactive, albeit detrimental, and energy metabolism stress, resulting in the destabilization of β-cell[50]. This cascade results in a reduction of Ca^{2+} ATPase in the Sarco/ER (SERCA), which is essential for transporting ER Ca^{2+}; the IP3 signaling pathway and a unique breakdown of ER homeostasis can trigger the same UPR pathway when large amounts of absorbed FFAs are detected[51].

Persistently elevated blood sugar, on the other hand, induces the elevation of islet amyloid polypeptides (IAAP) and catalyzes their reversible synthesis in β-cells. This induction results in IAAP with insulin misfolding accumulation and an enhancement in the establishment of reactive oxygen (ROS)-triggered oxidized protein degradation components[52]. These processes collectively damage ER, Ca^{2+} transport, pro-apoptotic signals, cleavage of glucose transport mRNA, and the development of interleukin-1 (IL-1), which is also supported by monocytes and reduces inflammation in proximal islets[24].

As previously noted, insulin secretion must always adapt to meet oxygen demands. A diminished insulin response in any of these tissues commonly enhances overall IR, ultimately contributing to T2DM. IR and pancreatic-cell failure occur simultaneously for the onset of T2DM[53]. Individuals who are overweight or obese may exhibit a certain level of IR, whereas diabetes generally manifests in those with reduced insulin emission relative to the levels of IR. However, insulin levels may indeed be excessive in some individuals; controlling glycemic is not always sufficient.

Obesity and T2DM have emerged as significant health challenges, driven by increasing screen time, reduced physical activity, excessive calorie consumption, and the consumption of sugary meals. The FDA has approved sodium-glucose cotransporter 2 (SGLT2) inhibitors for managing the side effects of diabetes mellitus[54]. While pharmaceuticals play a prominent role in treating T2D, post-marketing investigations have revealed adverse effects, including ketoacidosis, vaginal and urinary bladder infections, malignancies, bone disease, and foot and ankle amputations[55].

As a result, adopting a well-balanced diet and engaging in strength training to promote weight loss and reduce adiposity represent promising strategies to prevent or delay the onset of T2DM in individuals with slightly elevated risks[56]. The American Diabetes Association (ADA) has also recommended dietary counseling as a key component of the overall management of individuals with T2DM[57]. Low-carbohydrate, high-fat regimens have become widely embraced among individuals with obesity and diabetes, based on contemporary controlled trials[58]. Numerous researchers have discovered that KD plays a vital role in glycemic control and weight loss, proving to be an effective strategy for both obese and diabetic patients[58,59].

7. Result and discussion

7.1. Mechanisms linking obesity and diabetes

Being overweight is linked to a wide range of clinical, sociological, and psychological issues. Furthermore, IR has been correlated with both T2DM and obesity. The association between obesity and T2DM involves numerous pathways, with adipocyte accumulation being one of the determinants of this physiological syndrome. In addition to IR, a significant portion of overweight individuals do not exhibit hypoglycemia. Under normal physiological conditions, pancreatic β-cells within the islet of Langerhans secrete insulin adequately to counteract deficiency and maintain normal blood sugar sensitivity[60]. Mitochondrial dysfunction has been strongly associated with obesity or IR in both diabetes and pre-diabetic conditions, even among

those with a normal baseline for T2DM. Lymphocytes, unable to adapt to decreased insulin responsiveness due to the interplay between IR and obesity, contribute to the onset of T2DM.

7.2. Impact of low-carbohydrate diets on glucose metabolism and triglyceride levels in type 2 diabetes

The present status of the low-carbohydrate diet's efficacy for individuals with T2DM remains inconclusive (Table 4). The previous research often featured non-randomized study designs and provided short-term statistical information, thereby presenting challenges in establishing definitive conclusions. The sustained regulation of glycemia stands as an important parameter for individuals with T2DM and warrants both pharmaceutical intervention and appropriate dietary considerations. Different diet plans have been recommended for type 2 diabetes mellitus (Table 5). The management of T2DM involves not only the administration of oral anti-diabetic drugs but also necessitates the inclusion of insulin therapy.

A low-carbohydrate diet is anticipated to result in substantial declines in insulin levels, potentially leading to the discontinuation or reduction of pharmaceutical doses administered during T2DM oral antibiotic therapy[1]. Daly *et al.* assert that[74], particularly when compared to the pre-dietary phase, the model suggests an 85% decline in insulin availability. In the treatment of T2DM with oral anti-diabetic therapies, a low-carbohydrate diet has been observed to yield a similar effect. During the induction phase, this dietary concept not only lowered insulin and blood glucose levels over a 24-h period but also enhanced cell insulin sensitivity[75]. This effect is expected to exert a substantial impact, particularly on the reduction of body fat mass, as evident in both obese and healthy subjects[12].

The detection of HbA1c is widely acknowledged as an efficient screening factor for identifying the risk of serious health problems associated with T2DM. The implementation of a low-carbohydrate diet plays a pivotal role in influencing this characteristic across various cases, indicating effective glucose management[75]. Low-carbohydrate diets, characterized by a minimal percentage of carbohydrates, have shown promise in improving blood sugar control. Notably, when adhering to a dietary regimen providing sugars in a proportion serving approximately 25% of regular energy requirements, favorable and significant

Table 4. Studies on the effects of low carbohydrate diets in Type 2 diabetic patient

Duration	Amount of fat intake (per day, per diet, or limits)	Amount of carbohydrate intake (per day, per diet, or limits)	Lipid profile	HbA1c	Body weight	References
24 weeks	No data	≤20 g	HDL ↑	+	+	[61]
6 months	Fats account for nearly half of the energy requirement of calories consumed of 600 – 1800 kcal.	75 – 95 g	HDL ↑	+	+	[62]
4 months	No restrictions	<20 g	TRIGL ↓	+	+	[63]
14 months	No restrictions	21 g	TRIGL ↓	+	+	[64]
12 months	No data	≤40 g	No data	+	+	[65]
8 weeks	30% of daily energy demand	25% of daily energy demand	No data	+	+	[66]
3 months	70 – 75% of fat	5% CHO (≤30 g/day)	HDL↑TRIGL ↓	+	+	[67]
2 months	25 – 30% of fat	5 – 10% of CHO (≤ 25g/day)	No data	+	+	[68]
4 months	65% of fat	<10% of CHO	No data	+	+	[69]
3 weeks	60 – 70% of fat	5 – 10% of CHO (≤40 g/day)	HDL↑TRIGL ↓	+	+	[26]
12 months	Protein 1.5 g/unit of body weight and by pleasure to fat	Individualized carbohydrate restriction, resulting in a blood level of 0.5 – 3.0 mM/L of βHOB.	HDL↑TRIGL ↓	+	+	[70]
12 months	28% protein, 58% fat	CHO 14%,	No data	+	+	[71]
4 months	600 – 800 kcal/day, CHO <50 g	Protein: 0.8 – 1.2 g/kg of ideal body weight; fat: 10 g of olive oil each day	HDL↑TRIGL ↓	+	+	[72]
12 months	Hypocaloric. CHO 14% (50 g/d)	Protein 28%, fat 58% (10% saturated fat)	HDL↑TRIGL ↓	+	No data	[73]
6 months	Protein 1.5 g/unit of body weight and by pleasure to fat	CHO 20 g/day, total energy 2200 kcal/day	HDL↑TRIGL ↓	+	+	[18]

Notes: ↑: Increase; ↓: Decrease; +: Positive impact; CHO: Carbohydrate; HbA1c: Glycohemoglobin; HDL: High density lipoprotein; TRIGL: Triglycerides.

Table 5. Different diet plans for Type 2 diabetes mellitus: Recommendations and remarks

Policy	Comments and suggestions	References
A consensus report to adults on nutritional treatment for Type 2 diabetes as well as pre-diabetes	Limiting average carbohydrate consumption among diabetes patients really does have the strongest information for boosting glycemic control and may be accomplished through some kind of diversity of eating practices to suit particular requirements and desires. Low-average carbohydrate eating with low or very low-carbohydrate feeding patterns is really a practical solution for treating individuals with Type 2 diabetes.	[80]
Diabetes patients usually consume a low-carbohydrate diet—position statement	Lower-carbohydrate diets can be medically beneficial in dropping median blood sugar levels in people with Type-2 diabetes in the short-term, according to trustworthy research (up to 6 months). Low carbohydrate consumption aids in weight loss, and maintenance factors increase the risk of cardiovascular disease, such as high cholesterol and high blood pressure in diabetes patients.	[81]
Hyperglycemia and type 2 diabetes prevention—A compromise report	Nutritional interventions include the following: Low-carbohydrate, high-protein diets, as well as the Dietary Approaches to Stop Hypertension (DASH) diet, all help with glycemic management, but the Middle Eastern dietary trend appears to have many more advantages.	[57]
A health and care excellence guideline on carbohydrates and the control of diabetes	It is proposed that perhaps the dietary reference requirement for average carbohydrates should sustain around 50% of the total calorie intake for such an overall population.	[82]
A health and care excellence guideline on carbohydrates and the control of diabetes	Individuals with Type 2 diabetes might be presented with nutritional dietary options to help them lose weight while improving their overall glycemic control. Simple calorie restriction, fat lowering, absorption of carbohydrates with a reduced glycemic index instead of just a high glycemic index, and even dietary carbohydrate restriction are almost all choices (an advised intake of about 50 g per day seems to be safe for up to 6 months).	[81]
Diabetes UK nutritional guidelines for individuals with diabetes are scientifically proof-based recommendations on diabetes prevention and control	Low-carbohydrate diets could well be considered a good idea for losing weight in Type-2 diabetic patients when they are supported by a licensed physiotherapist, according to Diabetes UK 2011 guidelines.	[83]

changes in glycosylated hemoglobin have been observed. Conversely, no such improvements have been observed in scenarios involving a diet featuring carbohydrates constituting 50 – 56% of routine fuel requirements[66].

The positive benefits of such a low-carbohydrate diet on HbA1 concentration are supported by Kirk *et al.*'s meta-analysis[76]. Several complications commonly associated with T2DM involve alterations in blood lipids. An abnormal biosynthesis of both triglycerides and cholesterol in the liver contributes to the increased secretion of atherogenic lipids into plasma, leading to elevated low-density lipoprotein (LDL) and very LDL (VLDL) levels, along with decreased high-density lipoprotein (HDL) concentrations. These issues are consistently related to both diabetic pathogenesis and "metabolic disorders," potentially affecting the development of T2DM.

Atherogenic dyslipidemia significantly amplifies the overall risk of heart disease. Associated with heightened hyperglycemia and irregular lipid levels, this condition has been associated with glycation, oxygenation, methylation, and hydroxylation mechanisms, collectively contributing to long-term inflammation. Food choices exert a substantial impact on blood serum cholesterol. Notably, a distinct low-carbohydrate feeding pattern has been observed to positively influence blood lipid metabolism in individuals with T2DM. This effect is likely attributable to reduced serum levels of insulin in these individuals, promoting the same enzyme, hydroxymethylglutaryl lyase-CoA (HMG-CoA reductase), essential for ketone body production while limiting cholesterol synthesis in the liver.

Research highlights the fundamental relationship between insulin dysfunction and fatty acid metabolism. This relationship extends to cells responsible for producing molecules such as fats, hormones, and cytokines, thereby contributing to the heightened risk of chronic diseases in type 2 diabetic patients[77]. Intermittent energy restriction, a strategy that considerably restricts caloric intake, represents one common approach to addressing obesity. The strategy involves deriving energy from micronutrients in the following proportions: 10 – 20% proteins, 50 – 60% carbohydrates, and 10 – 30% lipids, a composition that aligns closely with conventional dietary presumption.

An increasing focus is placed on exploring various food types for achieving substantial weight loss. Notably, there is a growing emphasis on utilizing diverse food types for rapid weight loss (Table 1). The efficacy of the aforementioned

Table 6. Beneficial effects of low carbohydrate ketone diet in Type 2 diabetes mellitus

Design methods and aims	Results	References
Study design: Retrospective. Aim: to learn more about the experiences of patients with diabetes (T1DM or T2DM) who have already followed or are implementing the KD.	KD was administered for 6 – 19 months. The primary goal is to maintain blood sugar control, lower it, lose weight, and reverse diabetes. Respondents also reported benefits such as enhanced glycemic control, weight loss, and satisfaction.	[84]
Study design: Prospective. Aim: to explain features of the "LCHF" food habit and how affordable it is for particular Type 2 diabetic patients.	The great majority of people reported experiencing less hunger and urges. HbA1c (P<0.001), diabetic medicines (P<0.001), and weight (P<0.001) all seem to be socially challenging to track on LCHF diets.	[85]
Reliability of LCKD providing <30 g of carbohydrates each day for 90 days in obese diabetic women.	Substantial weight loss, HbA1c reductions, and blood pressure lowering, along with beneficial changes in blood lipids. Diabetes was completely reversed by LCKD.	[67]
Obese people with diabetes compared the advantages of a 20% carbohydrate vs. 55 – 60% carbohydrate diet on body weight, glycemic regulation, and cardiovascular effects.	HbA1c and body weight of participants who have observed a low-carbohydrate diet are improved. These sufferers have just a few more cardiovascular complications, but again, the controls are effective.	[62]
Obese and Type 2 diabetic individuals on LCKD with no calorie restriction were comparable to LGID, a low-calorie diet for 24 h.	In comparison to the LGID group, the LCKD survey showed significant changes in hemoglobin, HbA1c, body weight, and HDL cholesterol. Inside this LCKD group, 95.2% of patients seemed to have their diabetes medication minimized, compared with 62% in the LGID group.	[61]
The study was just to learn more about the impacts of MCCRD and LCKD on obese Type 2 diabetic people.	In comparison to the MCCRD group, participants in the LCKD group reported increased reductions in HbA1c levels and body weight.	[86]
After 6 months, obese subjects with only 39% incidence rates were compared with LCD and LFD.	LCD is over three times more efficient for losing weight. Starving blood glucose levels in LCD and LFD were lowered by 26 and 5%, respectively. LCDs have a significant impact on lipid profiles.	[87]
Enhanced KD on a very low-calorie ketogenic diet has quite a consequence on glucose concentrations in diabetic subjects.	There may be a significant negative relationship between circulatory ketone bodies and hepatic glucose output, demonstrating that larger ketones help people with diabetes maintain good glycemic control.	[63]

Abbreviations: KD: Ketone diet; LCD: Low-carbohydrate diet; LCHF: Low-carbohydrate high-fat; LCKD: Low-carbohydrate ketogenic diet; LFD: Low-fat diet; LGID: Low-glycemic index diet; MCCRD: Moderate-carbohydrate, calorie-restricted diet; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus.

diets is attributed not only to the quantities and sources of energy provided but also to the reduced fluid retention associated with a low-carbohydrate diet. It is hypothesized that adhering to such diets could facilitate weight loss in individuals with T2DM, especially during the initial stages. KD has demonstrated the ability to mitigate tumorigenesis in animal studies, attributed to the generation of adequate energy from excess sugar, which is crucial for tumor progression[78].

Elevated levels of estrogen (stemming from the heightened conversion of sex hormones by aromatase in adipocytes) could potentially be significant in hormone-dependent cancers (such as breast and ovarian cancers), even without variations in the dose of gestagens. This significance is particularly relevant in the context of defining overweight in women, where excess weight contributes to an increase in estrogen production (depending on the degree of androgen transformation by aromatase in fatty tissue). Estrogens induce an increase in free radical production, a factor among the mediators of mutagenesis. Consequently, accelerated cancerogenesis appears to be associated with reactive hypoglycemia, glucose intolerance, and higher serum expression of IL-6 and tumor necrosis factor, which are released by excessive fat cells in individuals with diabetes[79].

7.3. Beneficial effect of ketogenic diet in Type 2 diabetic patients

Different low carbohydrate ketogenic diets have been reported to be beneficial in type 2 diabetes mellitus (Table 6).

7.4. Risks associated with KD in type 2 diabetic patients

Despite the benefits observed with various low-carbohydrate diets in managing T2DM, due consideration should be given to the recognized risks associated with both short- and long-term practice of these diets. A potential side effect of low-carbohydrate diets is vitamin deficiency, with Vitamin C deficiency being particularly likely. T2DM is inherently associated with oxidative stress, and a notable drawback is the unavailability of polyphenols, such as flavonoids, which have been demonstrated to improve glucose production[88]. Addressing vitamin deficiencies is typically recommended, primarily given that a low-

carbohydrate diet lacking in vegetable fiber can adversely affect the growth of gut bacteria[89].

Consuming a low-carbohydrate diet for a brief duration has subsequently rendered the long-term implications of this dietary approach nearly impossible to predict. Furthermore, the increased serum uric acid concentration resulting from ketosis is another noteworthy concern[90], as it may foster hyperuricemia and accelerate nephropathy growth[91]. To facilitate uric acid excretion during the intervention, adequate water intake is imperative. In cases requiring it, sodium bicarbonate may also be administered to alkaline urine[90]. Renal impairment becomes more prevalent following the excessive intake of protein in one's diet. Studies indicate that patients with T2DM exhibit elevated ammonium content in their blood[82]. Lower protein intake has been demonstrated to delay glomerular filtration and prevent excessive kidney problems, a vital consideration for individuals with T2DM, often reported as a nephrological disorder[77]. It is worth mentioning that protein intake in these individuals should consistently be 0.8 g/kg/day, constituting <10% of the total energy input[92].

A low-carbohydrate diet may result in hypovitaminosis, rendering this dietary approach inadequate. It is advisable for the general population, and especially those with diabetes, to refrain from adopting such a diet[1]. Low-carbohydrate meals characterized by lower dietary fiber content can result in increased bile acid concentrations. This heightened concentration of bile acid, when converted to secondary bile acids, may contribute to the progression of congestion and potentially cause colorectal cancer[93]. To counteract potential nutritional deficiencies, Type 2 diabetic patients who adhere to low-carbohydrate diets receive fortification with vegetable fiber. In addition, during the initial weeks and months of a KD, reduced insulin levels induce increased natriuresis, kaliuresis, and diuresis. These effects lead to the loss of water and electrolytes in the body, contributing to electrolyte imbalance[94].

In Type 2 diabetic patients, a low-carbohydrate diet induces short-term alterations in bone remodeling and may contribute to the progression of both osteopenia and osteoporosis[95]. The prescribed heavy reliance on such a diet is especially crucial for postmenopausal Type 2 diabetic women, as their bone deterioration is further exacerbated by a limited carbohydrate intake. In addition, this dietary pattern increases the risk of gallstones. Although previously described benefits of several lipid profiles in what appears to be a low-carbohydrate or high-fat diet include a decreasing trend in cellulose fibers, these advantages have also been associated with an increment in LDL cholesterol levels both in patients with T2DM[96] and individuals without carbohydrate abnormalities[97].

8. Conclusion

For patients with obesity or T2DM, current recommendations support the utilization of low-carbohydrate diets as an alternative to traditional low-fat diets. Our review findings demonstrate the effectiveness of a KD in glycemic regulation for individuals with obesity and T2DM who are also hyperlipidemic. In addition to weight loss and improvements in lipid levels, there was a marked enhancement in HbA1c levels, accompanied by a significant reduction in the use of insulin or orally administered antidiabetic medications. It is important to note that these positive outcomes are typically observed after a short duration of utilizing this dietary pattern. While the potential for significant weight loss in patients with concurrent T2DM and obesity should be carefully considered, this type of diet does exhibit a positive influence on anthropometric parameters in the short-term, facilitating the effective reduction of excess weight. Low-carbohydrate diets have the potential to regulate glucose levels by minimizing insulin demand and reducing glycosylated hemoglobin content. Moreover, the findings of studies indicate that low-carbohydrate diets improve the serum lipid profile, notably triglyceride and HDL cholesterol levels, in individuals with T2DM. While the benefits of low-carbohydrate diets are evident, potential negative impacts need to be acknowledged. These food-restricted diets may increase the risk of mineral deficiency. Similarly, a shortage of fiber in combination with limited carbohydrates, especially in high-protein, low-carbohydrate diets, is associated with a heightened risk of renal dysfunction. An increased protein intake with low carbohydrate intake can contribute to water and electrolyte abnormalities, alongside modifications to osteoclast genesis, potentially resulting in osteopenia and osteoporosis. In assessing the advantages and disadvantages of a low-carbohydrate diet for individuals with T2DM, it is essential to recognize that there is no conclusive evidence supporting only one side of the argument.

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The authors declare that they have no competing interests.

Author contributions

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Ethics approval and consent to participate

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Data used in this work are available from the corresponding author on reasonable request.

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REVIEW ARTICLE

The significance of human papillomavirus integration in carcinogenesis and the development of specific diagnostics and countermeasures

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Abstract

Human papillomavirus (HPV) infection is associated with various tumors, notably in the cervix, oropharyngeal region, and anus. As the disease progresses, integration of the viral genome into the host genome is often observed, yet the means of integration and its intrinsic relationship to carcinogenesis remain unclear. To address this gap, novel sequencing technologies have been developed to enhance the accuracy, intuitiveness, and cost-effectiveness of identifying integration breakpoints. HPV genome integration is thought to induce imbalances or dysfunctions in gene expression, epigenetic changes, chromosomal translocation, and genetic instability. The precise mechanisms underlying the changes in gene expression caused by genome integration offer avenues for tumor risk prediction and prognosis assessment. Personalized precision medicine, grounded in the integration patterns unique to each patient, holds promise for cancer treatment. This review summarizes and discusses the current state of knowledge regarding the mechanisms, carcinogenesis, detection and analysis, and clinical significance of HPV integration. It synthesizes existing information to offer a comprehensive overview, potentially enhancing our understanding of HPV-related tumorigenesis and aiding in the development of more effective diagnostic and therapeutic strategies.

Keywords: Human papillomavirus; Integration mechanism; Carcinogenesis; Integration sites identification; Clinical significance

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1. Introduction

Globally, cervical cancer ranks as the fourth most common cancer among women, with an estimated 604,000 new cases reported in 2020. Out of the approximately 342,000 cervical cancer-related deaths in the same year, roughly 90% were concentrated in low- and middle-income countries[1]. In Africa and Southeast Asia, human papillomavirus (HPV)

stands out as the main infectious oncogenic pathogen, accounting for over 50% of infection-related cancers[2]. The identification of over 200 distinct HPV types has been a significant advancement in understanding this complex viral landscape. At present, 231 HPV reference types have been cataloged and submitted to the International HPV Reference Center (https://www.hpvcenter.se/human_reference_clones/). Five genera of HPV have been defined: *Alphapapillomavirus*, *Betapapillomavirus*, *Gammapapillomavirus*, *Mupapillomavirus*, and *Nupapillomavirus*. The alpha genus is particularly well-characterized, as a subset of these mucosal viruses has been classified as "high-risk" or carcinogenic. The World Health Organization (WHO) identifies at least 12 genotypes of HPV (genotype 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) as high-risk, with HPV16 and 18 being the most prevalent high-risk HPVs (HR-HPVs) worldwide[3]. Among the numerous HPV types, 20 display a notably higher prevalence in cases of cervical cancer when compared to women with normal cervical cytology. All HPVs infect epithelial cells, usually through the mucosal or cutaneous route. HR-HPVs are associated with various human mucosal surface cancers, including penile, vulvar, vaginal, anal, oropharyngeal, and cervical cancers (CC)[4]. Notably, it has been suggested that at least 90% of CC and over 70% of oropharyngeal cancers are associated with HR-HPV infection[5,6].

HPVs are non-enveloped, double-stranded circular DNA viruses characterized by a 7 – 8 kb genome comprising three distinct regions: The upstream regulatory region (URR), the early coding region (E), and the late coding region (L). Within the beta genus, HPVs lack the E5 open reading frame (ORF), and the size or position of ORFs can vary. However, HPVs generally contain highly conserved regions for replication (E1 and E2) and packaging (L1 and L2), with other genes displaying greater sequence diversity between genotypes[5]. E4 is thought to influence tropism and may affect virus release or transmission[7]. In the alpha genus, the E5, E6, and E7 proteins serve as accessory proteins supporting the HPV life cycle. They manipulate the balance of cell proliferation and differentiation while antagonizing innate immune pathways[8]. The E6 and E7 proteins of HR-HPVs interact with numerous proteins involved in cell cycle regulation, including p53 and pRB.

Persistent HPV infection is associated with cancer development, yet the precise mechanisms through which HPV induces carcinogenesis remain unclear. A critical oncogenic step involves the escape of HPV from immune system clearance and detection. Different proportions of T-helper (Th) cells, including Th17 cells, have been identified in different cervical lesion grades, exerting regulatory control over the immune microenvironment[9,10]. In the context of cervical carcinogenesis, the integration of the extrachromosomal viral genome into the host chromosome emerges as a common occurrence. Notably, in cervical cancer, HPV integration events can be detected in premalignant lesions, with the percentage of cells containing integrated HPV genome escalating with disease progression[11]. The integration event is, therefore, thought to transpire at a later stage in the progression of high-grade cervical dysplasia[12]. In cervical cancer cases, approximately 70% of HPV16-positive cases and almost all HPV18-associated cases exhibit detectable viral integration[5,13]. Similarly, about 75% of HPV16-associated oropharyngeal squamous cell carcinomas (OPSCC) and uterine cervical squamous cell carcinoma (UCSCC) display viral genome integration[14,15]. The significance of HPV integration is underscored by its potential utility as a biomarker for diagnosis, prognostic monitoring, survival prediction, and even in HPV-related cancer screening. Consequently, the integration status of HR-HPV has become a focal point in the realm of early diagnosis and precision treatment of cervical lesions.

It has been suggested that integration may confer a selective growth advantage to the virus and trigger host cell transformation through dysregulation of *E6/E7* viral oncogenes, resulting in an imbalance between cell proliferation and apoptosis and an increase in genetic instability[11,12]. However, it is crucial to recognize that *E6/E7* overexpression is not the sole factor underlying carcinogenesis. A series of structural and epigenetic changes in the host genome, resulting from viral genome integration, may also play an important role. Much remains to be elucidated about the mechanisms of integration and the ensuing patterns and alterations to the human genome. In this review, we will briefly describe the current understanding of the mechanism of HPV integration, the role of HPV in the neoplastic transformation and carcinogenesis process, recent advances in the detection and analysis of HPV integration, and the clinical significance of HPV integration in cancer prediction, prognostic evaluation, and the treatment for HPV-associated disease.

2. Mechanism of HPV integration

Little is currently understood about the mechanism through which the HPV genome integrates into the host genome. However, it is evident that this integration is an active process within the virus life cycle rather than an incidental or arbitrary occurrence. HPV integration exhibits a propensity to take place at random points throughout the human genome, with a notable preference for regions proximate to CpG sites, transcriptionally active regions, and common fragile sites[16]. It has been

suggested that HPV initially integrates into the host genome at random sites based on genome accessibility. However, over the course of extended carcinogenesis, there appears to be a propensity for integration to concentrate at specific loci, at which recurrent observations of integration have been made. This phenomenon may confer selective advantages to host cells, resulting in the formation of integration hotspots in different samples[17]. Several possible mechanisms have been proposed to elucidate HPV integration, including a DNA damage response, a looping model, and microhomology-mediated integration. Importantly, multiple integration mechanisms may concurrently operate. The following sections briefly introduce the principal mechanisms of integration that have been proposed.

2.1. DNA damage response

The frequency of integration is thought to be related to the degree of DNA damage, as the integration process necessitates the creation of double-strand breaks in both the viral and host DNA[18]. Notably, several proteins pivotal in DNA double-strand break repair pathways exhibit significant upregulation in various tumors. The integration into related genes further facilitates the insertion of the viral genome[19,20]. HPV infection and genome replication within cells induce the recruitment of excessive DNA damage factors, creating favorable conditions for the accidental integration of viral DNA[21]. Chromatin enriched in E2 and Brd4 is frequently situated proximal to common fragile sites and nucleic acid replication foci. This observation supports the theory that HPV exploits the host DNA repair mechanism, thus promoting its own replication[22]. Beyond its role in viral replication, E1 has been found to suppress the growth of host cells through a mechanism associated with the activation of DNA damage responses[23]. Consequently, E1 expression is hypothesized to promote HPV integration, with its interruption during integration resulting in the loss of the growth-suppressing properties associated with the E1 protein. Moreover, HPV16 E6*, the spliced isoform of E6, has been shown to elevate levels of reactive oxygen species, inducing chronic oxidative stress and cellular DNA damage. This process, in turn, increases the frequency of HPV integration, a relationship confirmed in both oral and cervical keratinocytes[24].

2.2. Looping model

In this model, HPV integration is mediated through DNA replication and recombination, providing insight into the structural aspects of HPV insertions flanked by host copy-number variations. Akagi *et al.*[25] observed a recurrent pattern of focal amplification and rearrangement adjacent to HPV integration sites in HPV-positive cancer cell lines. Their hypothesis posits that HPV integrants bridge discontinuous host sequences by connecting nicked sites. This connection leads to a focused amplification of breakpoints, including viral and flanking host sequences. Such amplification occurs through the replication of transient circular structures and circularized genomic segments. The subsequent recombination of distal free ends with noncontiguous sequences, coupled with repair processes, further explains the tandem insertion of HPV fragments. This tandem insertion exhibits one identical breakpoint shared across multiple fragments, while unique breakpoints manifest on the opposite side.

2.3. Microhomology-mediated integration

Zones of microhomology (MH) with a maximum length of 4 bp between the viral genome and the host genome can be detected in proximity to the integration sites. Initially, it was suggested that homologous stretches of DNA facilitate the annealing of partially dissociated strands, thereby facilitating recombination. Importantly, sequence homology at the integration site was posited as not strictly necessary[26]. Two probable mechanisms, FoSTeS[27] (fork stalling and template switching) and MMBIR (microhomology-mediated break-induced replication), have been identified in this context. Both mechanisms are triggered by local genomic instability and integration sites have been significantly enriched in several genomic instability-related elements, such as satellite and short interspersed nuclear element (SINE)-Alu repeats[17]. These findings suggest that HPV might leverage the zones of MH flanking the breakpoint to hijack the DNA repair pathway as a means of inserting the viral genome into the damaged host genome.

3. The role of HPV in cellular transformation and carcinogenesis

Integration alone proves insufficient to affect terminal cell differentiation or induce malignant transformation; additional alterations to host-cell genes and accompanying epigenetic modifications are required for tumorigenesis[28]. In the context of HPV-positive primary tumors, numerous structural variants and expression patterns, along with differential DNA methylation, have been associated with viral integration. Notably, many of these changes are shared between head-and-neck squamous cell carcinomas (HNSCC) and CC[13,19,29]. Earlier studies characterized viral integration patterns as Type I (a single integrated copy) or Type II (multiple tandem head-to-tail repeats of the viral genome). Recent studies, however, have confirmed that host-cell flanking sequences are often coamplified and rearranged. They have introduced a third integration type (Type III), where tandem copies of the HPV

genome are interspersed with cellular DNA, resembling a Brd4-dependent super-enhancer-like element[30]. The role of HPV integration in cell transformation and carcinogenesis is described below (Figure 1), considering both the direct and indirect effects of E6/E7 dysregulation. This exploration encompasses the influence of imbalanced cancer gene expression, epigenetic changes such as abnormal methylation, and changes in chromosomal structure.

3.1. E6/E7 oncoprotein overexpression

The abolition of E2 protein-mediated transcriptional repression of the E6 and E7 promoters leads to the overproduction of the E6 and E7 proteins, culminating in the inactivation of important cell cycle checkpoints and propelling cancer development[21]. Notably, screening tests for cervical cancer based on HPV *E6/E7* mRNA detection are commercially available[31]. The affinity of the E2 protein for various *E2* binding sites (*E2BSs*) is contingent on E2 concentration. At low concentrations, E2 binds to the high-affinity *E2BS1* (promoter-distal) site, activating the expression of *E6/E7*. In contrast, binding to *E2BS3* or *E2BS4* (promoter-proximal) results in the transcriptional repression of *E6/E7*[32,33]. During integration, the early 3' region of the virus is always deleted completely or truncated, resulting in the deletion or disruption of *E2* and the loss of the polyadenylation site. Conversely, the *E6* and *E7* genes remain intact[34]. Transcription in cells harboring integrated HPV DNA generates more stable virus-host fusion transcripts, conferring growth advantages on these cells compared to those harboring only extrachromosomal HPV[34-36]. Breakpoints also frequently occur in *E1*, influencing the downstream function of *E2*[12].

In CC samples in which the HPV-coiled coil domain-containing 106 (*CCDC106*) sequence integrates into the host genome, alternative splicing of *E6* occurs, leading to the generation of *E6*I*. In this context, *CCDC106* is strongly expressed, while there are low levels of p53 expression[37]. While E6 is generally implicated in p53 degradation in CC, the overproduction of *E6*I* inhibits this degradation, and the overexpression of *CCDC106* has an opposing effect. Consequently, the p53 oncogenic pathway is not only regulated by HPV oncogenes but is also influenced by alterations in host gene expression resulting from HPV integration. A more detailed understanding of the relationship between the splicing of *E6/E7* transcripts

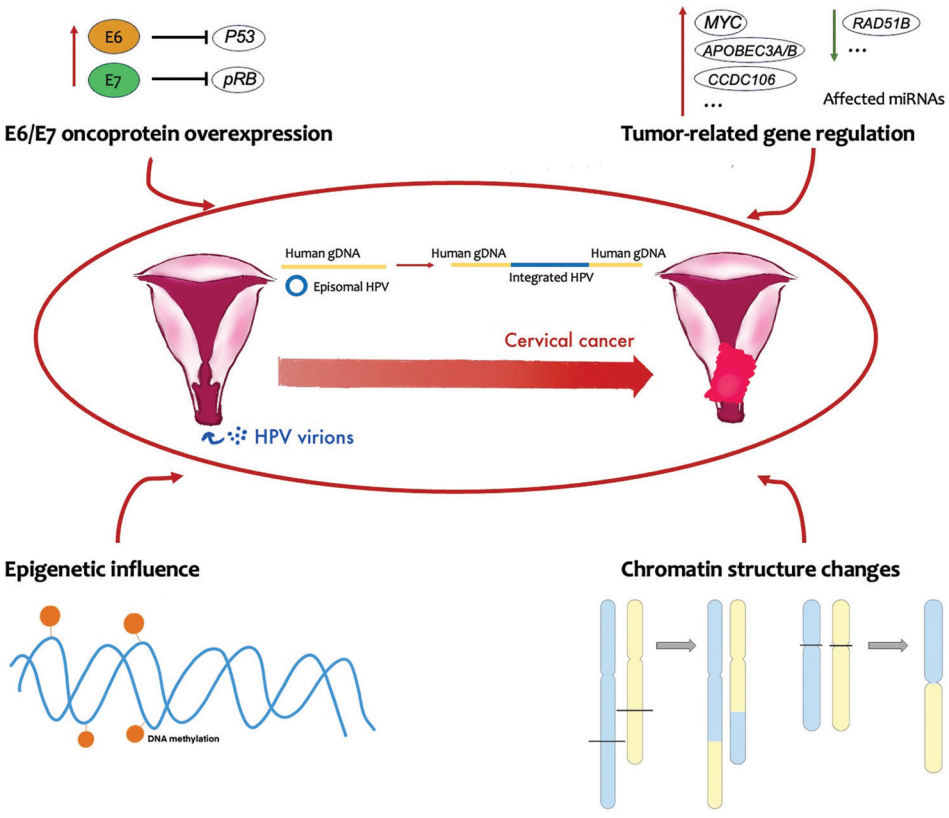

Figure 1. The role of HPV integration in the process of cell transformation and carcinogenesis.
Abbreviation: HPV: Human papillomavirus.

and HPV integration necessitates further investigation. In addition, as detailed below, other influences may affect the expression of these two proteins[38]. However, it is essential to note that breakpoints can occur in any region of the viral genome. Several studies have reported that differences in *E6/E7* transcript levels between samples of different histopathological grades or between different physical states of the viral genome are not statistically significant. These observations suggest that high levels of E6/E7 may not be imperative for tumorigenesis. Conversely, secondary genetic events, such as copy number variation, might be required to induce cancer[12,39].

3.2. Imbalance in the expression of tumor-related genes and the affected key cellular pathways

As mentioned above, the majority of the integration sites cluster in regions of open chromatin and common fragile sites. Identified as "integration hotspots," locations such as 3q28, 4q13.3, 8q24.21, 13q22.1, and 17q21 have been documented[4,26,40,41]. HPV integration disrupts host genes through various mechanisms, including genome rearrangements (amplifications, deletions, inversions, translocations, and others), intragenic insertions, and the introduction of promoters, splice acceptors, splice donors, and transcription termination elements[15]. The disruption of host genes by HPV integration can result in gene dysfunction through a complete loss of expression of the affected genes at the RNA or protein level[42]. Integration within 50 kb of known oncogenes or tumor repressor genes can occur, often resulting in the downregulation of tumor suppressor genes and the upregulation of proto-oncogenes[41]. DNA from HPV16 or 18 has been detected downstream, upstream, and within the *MYC* gene (8q24.21 integration hotspot) at distances ranging from 0 to 513 kb from this gene. This results in *MYC* overexpression at both the mRNA and protein levels, suggesting a potential contribution of the *MYC* activation triggered by the insertion of HPV viral DNA to cervical carcinogenesis[28,43]. In HeLa cells, the integrated HPV fragment is 3300 kb away from the 8q24.22 region in the linear distance, but the integrated HPV DNA can be brought into the close vicinity of the 8q24.22 region by chromatin folding, which is required for colocalization of the *MYC* locus and 8q24.22 region[44]. *MYC* and *PDL1* are also frequently integrated and overexpressed in HNSCC[15,45].

The proteins belonging to the apolipoprotein B mRNA-editing catalytic (APOBEC) polypeptide family increase the likelihood of DNA breaks. Levels of the APOBEC3B polypeptides are significantly higher in HPV-positive HNSCCs than in HPV-negative HNSCCs[46], and *APOBEC3A* is overexpressed in biopsy samples from oropharyngeal cancer displaying HPV16 integration[20]. A study of 25 HNSCC cases exhibiting HPV integration identified integration sites within the gene encoding the DNA repair protein RAD51 homolog 2 (RAD51B), resulting in an amplified chimeric segment encoding a non-functional RAD51B protein[19]. Li *et al.*[47] found that the genes repeatedly targeted by HPV integration were enriched not only in genes related to DNA repair pathways but also in genes associated with the pathways of xenobiotics metabolism by cytochrome P450, chemical carcinogenesis, and steroid hormone biosynthesis. Integration ratios increased with the progression of CC. The *ERBB2* gene (which enhances the MAPK and PI3CA pathways) is another reported site of HPV integration[13]. It has been suggested that even though integration can occur at multiple separate loci in tumor genomes, there is usually only one transcriptionally active locus[48]. MicroRNAs (miRNAs) close to integration sites may also be influenced. One study identified 47 integration sites and showed that 32 of these sites were within 3 Mb of a miRNA locus, and 19 of the 75 affected miRNAs were associated with tumors, influencing cell proliferation, differentiation, and apoptosis[26].

3.3. Epigenetic changes

Epigenetic changes may occur in both the HPV and host genomes, providing integrated viruses with a selective advantage. Therefore, it is necessary to consider both integration and epigenetic changes[4]. Most studies on epigenetic changes to date have focused on methylation. Epigenetic and genetic alterations to *E2BSs* in the HPV16 URR (with *E2BS3/4* becoming more methylated than *E2BS1* in CC) may also block the suppressive function of *E2*. A remarkably high degree of heterogeneity has been observed for URR methylation levels in squamous cell carcinoma samples displaying HPV genome integration[32,49]. The internal copies of the viral genome are always epigenetically silenced, and Type 2 integration events are associated with greater hypermethylation of the long control region[50]. The integration of HPV16 viral DNA into the host genome is associated with hypermethylation in the *L1/L2* and *E2* regions in vulval intraepithelial neoplasia[51]. In one study, the methylation status of the integrated HPV DNA was found to be correlated with the methylation status of the host genome close to the integration site in three HNSCC cell lines[52]. Brd4-enriched transcriptional regulatory hubs have been identified in cells displaying HPV16 genome integration. These hubs act as a super-enhancer-like element, promoting the transcription of *E6* and *E7*[30,53]. The expression of the *E7* gene in HR-HPVs leads to the extensive epigenetic reprogramming of cells. In HPV16-E7 expressing cells and HPV16-positive cervical intraepithelial neoplasia (CIN) specimens, levels

of H3K27me3, an important class of transcriptional repressive post-translational modifications, may decrease, while levels of the H3K27-specific demethylases (KDM6A and KDM6B) increase[54]. The induction of transcription in the genes encoding these enzymes is reminiscent of what occurs in the wound-healing model, suggesting that HPV tends to maintain a wound-healing state favorable for the viral life cycle. In HNSCC samples, HPV integration sites also colocalize with H3K27ac (active histone marks) enhancers upstream from several genes related to HNSCC tumorigenesis, such as *TP63*, *FOXE1*, *NOTCH1*, and *EGFL7*[55].

3.4. DNA instability and changes in chromatin structure

Integration of the HPV genome results in structural changes in the host genome at the insertion sites. HPV integration is frequently accompanied by somatic copy number variations, including focal amplifications, deletions, and intra and interchromosomal rearrangements[12]. For example, interchromosomal translocation between chromosomes 3 and 13, resulting from HPV insertion in HNSCCs, leads to elevated levels of RNA for *KLF5*, *TP63*, and *TPRG1*[19]. In cell lines displaying HPV integration, chromatin interactions between the integrated viral genome and host chromosomes occur both 500 kb away from integration sites, potentially causing dysregulation of host genes[56]. In a prospective cohort of 272 patients with annotated CC, the *MACROD2* gene emerged as a hotspot for viral genome integration. The intronic integration site in *MACROD2* may underlie genomic instability, given its association with a common fragile site[57].

4. Methods for detecting, identifying, and analyzing HPV integration sites

Technological advancements have introduced more efficient and accurate methods for the identification of viral integration sites. Traditional methods for the identification of integration sites relied on fluorescent in situ hybridization (FISH) and immunohistochemistry. Morphological approaches remain the preferred choice for the visually localizing integration sites in cells, enabling the observation of the state and location of viral DNA and its interaction with chromatin[58]. However, the genes affected by integration can only be identified by molecular methods. The following sections provide a brief description of the molecular methods available for detecting, identifying, and analyzing integration sites.

4.1. Polymerase chain reaction-based techniques

Polymerase chain reaction (PCR)-based techniques can be classified into two groups depending on whether the amplification product is derived from the transcriptome or the nucleic acid of HPV itself: those that are RNA-based and those that are DNA-based. One such RNA-based technique is an amplification of papillomavirus oncogene transcripts (APOT), designed to detect HPV-derived fusion transcripts. These transcripts undergo reverse transcription, and the assay relies on a 3'-rapid amplification of cDNA ends in a nested PCR format. Utilizing HPV E7 primers as forward primers, an adapter primer complementary to the linker sequence serves as the first reverse primer, followed by a shifted primer as the second nested primer[59]. The assay is universally adopted for detecting viral-cellular fusion transcripts and analyzing integration sites carrying transcriptionally active viral genes[40].

Unlike RNA-based methods, which exclusively detect transcriptionally active integrants, DNA-based methods detect all integrated virus genomes, regardless of their transcriptional activity. Integrated papillomavirus sequences have been detected through ligation-mediated PCR (DIPS-PCR) to pinpoint the locus of HPV genome integration within the DNA[60]. This method involves the ligation of endonuclease-specific double-stranded adapters, followed by linear amplification with specific primers, resulting in the production of DNA fragments containing HPV. Finally, double-stranded PCR products are generated through PCR amplification with HPV-specific and adapter-specific primers.

4.2. Next-generation sequencing (NGS)-based techniques

At present, NGS stands as the principal method for the accurate identification of insertion sites. The NGS approaches include unbiased, untargeted techniques, such as whole-genome sequencing (WGS) and RNA sequencing (RNA-seq), as well as the utilization of specific probes and primers for targeted enrichment. Different probes can be designed to capture different HPV genomes. Subsequently, NGS is performed, and information about integration is derived from HPV-enriched DNA libraries. This method is considered unbiased and applicable to any sequence containing the HPV genome, be it episomal or integrated into a chromosome[61]. The integration analysis can be further enriched by combining DNA and RNA sequencing, offering comprehensive information about HPV integration at both genomic and transcriptomic levels[19,62]. A previous review discussed the various applications of NGS in HPV integration studies[63].

The high-throughput viral integration detection method, initially developed for detecting HBV integration[64], utilizes viral probes to capture inserted viral genome fragments in the host genome. Employing an effective bioinformatic

pipeline with a pair-assembling strategy, integration sites are identified through data analysis. This approach has proven successful in several studies for pinpointing meaningful integration sites[65]. Another strategy, tagging, enrichment, and NGS of HPV16 (TEN16), relies on targeted next-generation DNA sequencing[66]. The process involves Nextera *in vitro* transposition for DNA fragmentation and adapter-tagging, followed by multiplex PCR with HPV16-specific primers for HPV16 DNA enrichment. This approach successfully identified 75 HPV16 integration sites in a mixed tumor sample pool. RNA-sequencing stands out as another method of choice for detecting viral-host fusion transcripts. Koneva *et al.*[67] successfully identified 320 integration breakpoints distributed across the human and HPV genomes using RNA-seq data from 84 HNSCC tumors. In addition, whole-exome sequencing and high-throughput chromosome conformation capture sequencing have been adapted for the identification of HPV integration sites[56,65,68].

Parsing the NGS data is crucial for the identification of integration sites, and various novel bioinformatic pipelines have been developed to process the data to obtain the necessary information. ViFi, a computational approach in 2018, utilizes phylogenetic methods combined with reference-based read mapping for calling integration sites[69]. ViFi achieves not only high precision and sensitivity in identifying fusion reads but also detects circular extrachromosomal DNA (ecDNA) human-viral fusion structures. SearcHPV is another novel pipeline based on targeted capture technology, specifically designed for the analysis of targeted capture DNA sequencing data. Notably, SearcHPV demonstrates higher sensitivity and specificity for detecting HPV-host integration sites compared to VirusFinder and VirusSeq, two integration callers developed earlier. It is important to acknowledge that a significant limitation of SearcHPV is its validation on only three samples[70]. In addition to SearcHPV, deep-learning models such as DeepHPV have been designed for predicting HPV integration sites[71]. A comprehensive database is essential for systematic searches, comparisons, and analyses of viral integration sites. The Viral Integration Site Database was developed as a user-friendly web tool for browsing, searching, analyzing, visualizing, and downloading data[72]. The utilization of well-organized, comprehensive, and continuously updated databases, along with the availability of various evolving intelligent analysis pipelines, has significantly alleviated the burden of analyzing large amounts of sequencing data in advanced biomedical research.

4.3. Third-generation sequencing-based techniques

Short reads are suitable for investigating specific structural variations at insertion sites, such as deletions or chromosomal translocations. However, they lack the capacity to reveal more extensive changes, such as changes in gene copy number resulting from the integration of the HPV genome into the host genome. The development of long-read single-molecule sequencing technologies by Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) has introduced new possibilities for the application of direct nucleic acid enrichment tools. Long-read sequencing enables the elucidation of complex gene rearrangements, and Nanopore analysis requires smaller amounts of sequencing data compared to Illumina methods while maintaining similar accuracy[73,74].

One study successfully identified four new candidate target genes for CC by integrating multi-omics data from The Cancer Genome Atlas with patient-matched long-read sequencing of HPV integration sites. The identified genes are *BNC1*, *RSBN1*, *USP36*, and *TAOK3*. The study employed the HPV-targeted PacBio long-read sequencing method and validated the potential involvement of these genes in tumorigenesis by assessing their expression levels. It is essential to note that the study had a limited sample size, including samples from only eight women, and as such, its findings may not be broadly representative[75].

Pooled CRISPR inverse PCR sequencing (PCIP-seq) is a method employing a pool of CRISPR guide RNAs for selective cleavage of circular DNA fragments carrying viral DNA. Subsequently, reverse long-fragment PCR and multiplex sequencing are performed using the MinION platform. The DNA is then sheared into approximately 8 kb fragments, followed by intramolecular ligation and removal of remaining linear DNA through digestion. To selectively linearize circular DNA containing viral sequences, specific regions of the HPV18 genome are targeted for selective CRISPR-mediated cleavage, and PCR amplification is performed before sequencing. The identified insertion sites support the hypothesis that rearrangements occur through a "looping" mechanism, as the two sets of breaks share a common breakpoint[76].

Xdrop technology is a novel microfluidics system designed for targeted enrichment in low-input and long-fragment DNA. The technology relies on the isolation of long DNA fragments within millions of droplets, wherein the droplets containing a fluorescently labeled target sequence of interest are sorted using fluorescence-activated cell sorting. The sorted DNA fragments undergo amplification through multiple displacement amplification (MDA) and are subsequently subjected to sequencing. This approach successfully resolved three HPV18-chr8 integration sites at base-pair resolution[77]. Table 1 summarizes the studies conducted on the identification of HPV integration sites for various types of

Table 1. Methods used to identify HPV integration sites across various cancers in studies published from 2020 onwards

Cancer type	Technology	Method (s) used	Number of samples	Infected HPV types	Number of integration sites identified	Genes into which HPV integrates at high frequency	References
Cervical cancer	FISH	Dual-color FISH	96	16, 18	N/A (62 cases of cervical cancer displayed HPV integration)	*FHIT*, *MYC*	[58]
	2nd-generation sequencing	HPV double capture and Illumina sequencing	272	16, 18, 31, 33, 39, 42, 45, 52, 56, 58, 59, 68, 70, 73, 82	308	*MACROD2*, *MIPOLA/TTC6*, *TP63*	[57,94]
		Virus capture sequencing	1,466	6, 11, 16, 18, 31, 33, 34, 35, 39, 45, 52, 56, 58, 59, 66, 68, 69, 82, etc	1,002 in 24.8% of non-cancer samples, 588 in 38.0% of precancer samples, and 1597 in 69.0% of cancer samples	*LINC00392*, *LRP1B*, *DIAPH 2*, *PROS1*, *LSP1P5*, *ANKRD26P1*, *FHIT*, *MACROD2*, *ERBB2*, *RREB1* and more (24 hotspots identified)	[95]
		Tagmentation-assisted multiplex PCR enrichment sequencing (TaME-seq2)	36	51, 52, 59	1	N/A	[96]
	3rd-generation sequencing	HPV DNA hybridization capture plus Illumina sequencing and nanopore sequencing, FISH used to visualize the integration site	1	70	1	*BCL11B*	[97]
		Nanopore sequencing and Illumina sequencing	1	16	83	Intergenic region of *CHMP48* and *RALY-AS1*	[73]
		HPV-enriched DNA for PacBio sequencing with matched multi-omics data on TCGA	8	16	267	*BNC1*, *RSBN1*, *USP36*, and *TAOK3*	[75]
		Nanopore sequencing	16	16, 58	63	*LINC00290*, *LINC02500*, and *LENG9*	[86]
		Nanopore sequencing	1	16, 35	448 in CaSki and 60 in clinical samples	N/A	[98]
	3rd-generation sequencing combined CRISPR	Pooled CRISPR inverse PCR sequencing (PCIP-seq), nanopore sequencing	2	18	74	*NISCH*, *PBRM1*	[76]
	3rd-generation sequencing combined with microfluidics	DNA enriched through microfluidics, PacBio sequencing, nanopore sequencing and Illumina sequencing	0 clinical sample, HeLa cell DNA used	18	4	N/A	[77]

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Table 1. (*Continued*)

Cancer type	Technology	Method (s) used	Number of samples	Infected HPV types	Number of integration sites identified	Genes into which HPV integrates at high frequency	References
	Multi-omics	Whole-genome, RNA, chromatin immunoprecipitation and high-throughput chromosome conformation capture (Hi-C) sequencing, HIVID	61	16, 18, 52, 53, 56, 58	109	*CCDC106*	[37,65]
		HPV-capture sequencing, RNA sequencing, and whole-genome bisulfite sequencing	50	16, 18, 31, 33, 45, 56, 58, 59, 73, 82	1,470	*LINC00486*, *LINC02425*, *LLPH*, *PROS1*, *KLF5*, *LINC00392*, *MIR205HG*, *NRG1*	[99]
		High-throughput HPV capture sequencing, whole-exome sequencing, transcriptome sequencing, proteomics, phosphoproteomics, and single-cell RNA sequencing	106	6, 16, 18, 31, 33, 34, 45, 52, 58, 66	762	*FHIT*, *TP63*, *KLF5*, *RASSF6*, *LRBA*, *TCERG1L*, and more (21 recurrent integration regions identified)	[100]
		Hi-C sequencing, whole-genome sequencing, RNA sequencing, ChIP-seq and CUT&Tag, virus capture second-generation sequencing, nanopore whole-genome and ecDNA sequencing	0 clinical sample, 9 cell lines	16,18	135	N/A	[101]
Small cell cervical carcinoma	Second-generation sequencing	High-throughput HPV capture sequencing, whole-genome sequencing, whole-transcriptome sequencing, and OncoScan microarrays	214	16, 18, 31	381	*MYC*, *SOX*, *NR4A*, *ANKRD*, *CEA* family genes	[102]
Endocervical adenocarcinomas	Second-generation sequencing	Whole-genome sequencing and whole-exome sequencing	20	16, 18, 21, 27, 40, 51, 52, 56, 57, 59, 61, 62, 81, 123, 127, etc.	N/A (1,036 genes with somatic mutations in samples presenting HPV integration)	N/A (*PIK3CA*, *KRAS*, *TRAPPC12*, *NDN*, GOLGA6L4, and *BAIAP3* predicted as driver genes)	[103]
Mucoepidermoid carcinomas	Second-generation sequencing	HPV16 capture-based targeted DNA sequencing and RNA sequencing	48	16	22	N/A (13 host genes displaying HPV integration)	[104]
Head and neck squamous cell carcinoma	Second-generation sequencing	Double capture-HPV method followed by Illumina sequencing	80	16, 26, 33, 35, 56	267	*PDL1*/*PDL2*/*PLGRKT*, *MYC*/*PVT1*, *MACROD2*, *KLF5*/*KLF12*	[45]

(*Cont'd...*)

Table 1. (*Continued*)

Cancer type	Technology	Method (s) used	Number of samples	Infected HPV types	Number of integration sites identified	Genes into which HPV integrates at high frequency	References
Oropharyngeal cancer and gastric cancer	Sanger sequencing	3' rapid amplification of cDNA ends for the detection of *E6/E7* fusion RNA	2 oropharyngeal cancer tissues and 1 gastric cancer tissue	16	1 (in oropharyngeal cancer)	*KIAA0825*	[105]
Oropharyngeal cancer	Second-generation sequencing	Whole-genome sequencing	105	16, 18, 33, 35, 59, 69	874	*SOX2*, *TP63*, *FGFR*, *MYC*, *CD274*	[15]
	Third-generation sequencing	Long-read sequencing (PacBio HiFi and nanopore sequencing) and whole-genome sequencing	4-cell lines and 105 clinical samples	16, 18	N/A (focus on resolving the structures of genomic rearrangements flanking integration sites)	*MYC*	[106]
Penile squamous cell carcinoma	Second-generation sequencing	HIVID	108	6, 16, 18, 33, 44, 51, 56, 58, 62, 66, 68	2,252	*KLF5*, *LRP1B*, *KLF12*, *CADM2*, *CEP19*, *CSMD1*, *NRROS*, and more (in total 24 genes with≥3 events)	[107]
Anal squamous cell carcinoma	Second-generation sequencing	Whole-exome sequencing	72	6, 16, 18	33	N/A (associated with CNVs)	[108]

Abbreviations: ChIP-seq: Chromatin immunoprecipitation sequencing; ecDNA: Circular extrachromosomal DNA; FISH: Fluorescence *in situ* hybridization; HIVID: High-throughput viral integration detection; HPV: Human papillomavirus; PCR: Polymerase chain reaction.

tumors since 2020, with a predominant focus on cervical cancer in most publications. Emerging studies employing multi-omics techniques (such as genomics, proteomics, transcriptomics, and epigenomics) hold promise for offering multidimensional, integrative approaches to unveil new associations between biological entities and relevant biomarkers.

5. Clinical significance

In the latest WHO guidelines for the screening and treatment of cervical precancer lesions aimed at preventing CC, HPV DNA testing is recommended as the principal screening approach for diagnosis and treatment[78]. However, genotyping alone proves effective solely in CC prevention. A more clinically relevant strategy involves a combination of genotyping, evaluation of integration status and sites, analyses of epigenetic changes, and examination of tumor-related immune molecules (such as HLA-G)[79]. It is important to note that nonvalent HPV vaccines, such as Gardasil 9, covering HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58, hold the potential to prevent up to 90% of all cases of cervical cancer. While preventive vaccination against HPV, especially HPV16 and HPV18, proves effective in halting the spread of HPV-related cancers, there is a pressing need to expand vaccination efforts in developing countries. Moreover, the development of new treatments, such as therapeutic vaccines, remains imperative for addressing the unvaccinated aging population suspected of HPV infection. Analyzing the landscape of HPV integration patterns has unveiled clinical and molecular associations, along with altered signaling pathways that could serve as predictive, prognostic, or therapeutic biomarkers for HPV-associated mucosal neoplasia[80].

5.1. Cancer risk assessment and predictive markers

Many studies have consistently demonstrated that HPV integration is a prevalent event in the initial stages of cervical carcinogenesis (such as CIN1). As the disease progresses from CIN to cancer, both the rate and number of integration events increase. This underscores the potential value of these markers as predictors of cancer progression[17]. The molecular profile of entire genomes displaying HPV integration can serve as an indicator of the duration of CIN. Notably, utilizing a methylation marker panel has proven to be a highly sensitive method for screening CIN undergoing transformation. In a study conducted by Liu *et al.*[81], it was observed that methylated CpG sites frequently co-occurred with integration sites in the HPV16 genome. Clinical samples validated that a panel of CpG sites (nt5606, nt5609, nt5615, and nt5378)

could potentially serve as biomarkers for the diagnosis of CC. Assessing the methylation status of *MAL* and *miR-124-2* has demonstrated similar efficacy to cytological tests in detecting CIN2+ in a study involving over 500 HR-HPV-positive women[82]. Another study revealed that the hypermethylation of four genes, including *hsa-miR-124,* was meaningful for predicting precursor lesions in CC[83]. As *E2* is systematically disrupted during integration, the *E2*/*E6* mRNA ratio can provide valuable information about the integration status. An *E2*/*E6* ratio below 1 is indicative of integrated viral DNA. In a study of 248 patients diagnosed with only HPV16 infection, it was found that an HPV16-*E2*/*E6* ≤0.6471 or relative levels of *POU5F1B* mRNA ≥1.0310 in cervical exfoliated cells could reliably predict CIN2+ tumors[84]. *POU5F1B* is located on chromosome 8q24, a frequent site of HPV integration. However, it is important to note that hybrid virus-human episomes may also occur, and in such cases, the *E2*/*E6* ratio is around 0.5, even in the absence of integration. Thus, the *E2*/*E6* ratio may be irrelevant if *E2* is not the principal gene displaying integration and disruption[85]. If effective biomarkers can be identified and utilized in clinical practice, it may be possible to avoid invasive colposcopy in cases where the risk of CC is sufficiently low, indicated by low levels of molecular aberrations and negative results for methylation markers[38].

5.2. Prognostic markers

Determining the physical status of HPV and identifying integration sites and affected genes could facilitate individualized patient follow-up, evaluation of disease-free survival, and early clinical diagnosis of recurrent disease. HPV-positive HNSCC generally has a more favorable prognosis than HPV-negative HNSCC, and patients with integration-negative tumors exhibit better survival than those with integration-positive tumors, especially coupled with a strong HPV-specific immune response[6,67]. Multiple integration events are significantly associated with poor prognosis[86]. Individualized tests targeting the junction between viral and cellular genomes, when combined with cytology, exhibit a lower false positive rate for predicting recurrence compared to standard HPV DNA/cytology cotesting (18.2% and 12.1%, respectively). However, this strategy lacks sensitivity, detecting integration in only 10.8% of patients[87]. Shin *et al.*[88] observed lower disease-free survival in CC patients with completely integrated HPV genomes compared to those with both integrated and episomal HPV, supporting the use of integration patterns as prognostic indicators. Combining HPV integration with proteomic data identified RAB2A as a potential predictive biomarker for longitudinal surveillance and prognosis evaluation in HPV-positive patients. RAB2A levels are significantly elevated in primary cancers compared to adjacent normal tissues, and high RAB2A levels are associated with poor survival[89].

5.3. Therapeutic options

In addition to traditional treatments such as surgery, radiotherapy, and chemotherapy, an increasing number of clinical trials now concentrate on addressing the carcinogenic factors associated with HPV infection (Table 2). Potential therapeutic methods related to virus-host interactions include knocking out integrated HR-HPV DNA, inhibiting early virus gene transcription, utilizing epigenetic methods (demethylation agents), and employing specific drugs targeting identified genomic alterations.

Decreasing or eliminating viral transcripts through vaccination is a potential approach that could complement traditional chemoradiotherapy methods for HPV-associated tumors. Therapeutic vaccines targeting HPV E6/E7 have already been developed, demonstrating the ability to activate strong cytotoxic T-lymphocyte responses[90]. For instance, Advaxis (ADXS11-001, Advaxis Inc., New Jersey, USA), expressing the HPV16-E7 antigen, exhibits promising therapeutic efficacy[91]. In addition, RNA-based therapies aimed at silencing *E6/E7*, including short-interfering RNA, CRISPR gene editing, miRNAs, long non-coding RNAs, and mRNA-based HPV vaccines, have also been developed.

The feasibility of translating these technologies into clinically relevant modes of treatment for HPV-derived cancers and potentially other virus-driven human cancers has been clinically or experimentally validated[92]. In W12 20861 cells (characterized by tandemly repeated HPV16 integration), the use of bromodomain inhibitors to displace Brd4, disrupting super-enhancer function, has demonstrated a decrease in the transcription of HPV16 *E6/E7* and the induction of cellular senescence. This suggests that cancer cells harboring integrated HPV could be a potential treatment target[53]. Precision medicine is also applicable to certain gene products already shown to be altered in cancer. For instance, PI3K inhibitors are currently under clinical trials for treating HNSCC[12]. In CC, *ERBB2* may be activated by HPV integration in some patients, making them potential candidates for inclusion in clinical trials of *ERBB2* inhibitors[93].

Table 2. Clinical trials with novel therapies for HPV-associated cancers

Clinical trial No.	Cancer type	Name	Principle	Treatment effect	Status	References
NCT05686226	Metastatic or refractory/ recurrent HPV16+cancer	E7 TCR-engineered T cells	Infused T cells express the HPV16 E7 TCR and bind the $E7_{11-19}$-HLA-A*02:01	Robust tumor regression was observed with objective clinical responses in 6 of 12 patients, including four of eight patients with anti-PD-1 refractory disease (in phase I clinical trials)	Phase II recruiting	[109]
NCT02280811	Metastatic or refractory/ recurrent HPV16+cancer	E6 TCR-engineered T cells	Infused T cells express the HPV16 E6 TCR and bind the $E6_{29-38}$-HLA-A*02:01	Two of the 12 patients achieved objective tumor responses. A patient with three lung metastases experienced complete regression of one tumor and partial regression of the other two	Phase I/II completed	[110]
NCT02379520	High-risk HPV-infected cancer	HPV16/18 E6/E7-specific T lymphocytes	HPV16/18 E6/E7-specific T cells	Unknown	Phase I	[111]
NCT04713046	Metastatic or refractory/ recurrent HPV16+cancer	HPV-specific T cells	CD8 depleted peripheral blood cells taken from related donors vaccinated against HPV16	Unknown	Phase I/II	https://clinicaltrials.gov/study/NCT04713046
NCT03912831	Refractory/recurrent HPV16+cancer	KITE-439	HPV16 E7 T-cell receptor-engineered T-cells	Unknown	Phase I	https://clinicaltrials.gov/study/NCT03912831
NCT04672980	Metastatic or refractory/ recurrent HPV16+cancer	RTX-321	RTX™-321 is an engineered antigen-presenting red blood cell that expresses human leukocyte antigen (HLA)-A*02:01 with human papillomavirus (HPV) 16 E7 $peptide_{11-19}$ (HLA-A2-HPV), 4-1BBL, and IL-12	RTX-321 activates HPV-specific T cells and promotes effector function *in vitro*	Phase I	[112]
NCT02853604	Advanced cervical cancer	ADXS11-001	Immunotherapy based on *Listeria monocytogenes* bioengineered to secrete a tLLO-HPV-16E7 fusion protein	Better overall survival was observed in ADXS11-001 treatment groups in a Phase 2 trial	Phase III	[113]
NCT03946358	HPV-positive anal cancer, head-and-neck carcinoma, or cervical and vulvar carcinoma	UCPVax and atezolizumab	UCPVax is a CD4 Th1-inducing anti-cancer vaccine composed of two separate peptides, UCP2 and UCP4, derived from telomerase. Atezolizumab is a PD-L1 inhibitor	The UCPVax vaccine is currently under evaluation in a multicenter phase I/II study in non-small cell lung cancer (NSCLC) (NCT2818426). It appears to be safe and immunogenic	Phase II	[114,115]

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Table 2. (*Continued*)

Clinical trial No.	Cancer type	Name	Principle	Treatment effect	Status	References
NCT03618953	Recurrent or metastatic HPV-associated tumor	MG1-E6E7 and Ad-E6E7	Oncolytic Maraba virus with Adenovirus vaccine both expressing mutant HPV16/18 E6 and E7	Induction of multifunctional, specific antitumor $CD8^+$T cells capable of eradicating advanced tumors in a durable manner in mice	Phase I	[116,117]
NCT05108870	HPV 16^+head and neck squamous cell carcinoma of the oropharynx	HB-201 and HB-202	Engineered replication-competent arenavirus vectors expressing HPV16 E7 and E6 genes	A single injection of HB-201 or HB-202 monotherapy is highly immunogenic in several patients, as demonstrated by an increase in inflammatory cytokines/ chemokines and antigen-specific $CD8^+$T cell responses	Phase I/II recruiting	[118]
NCT03978689	$HPV16^+$Recurrent/ metastatic head-and-neck squamous cell carcinoma	CUE-101	CUE-101 is an Fc fusion protein composed of a human leukocyte antigen (HLA) complex, an HPV16 E7 peptide epitope, reduced-affinity human IL2 molecules, and an effector attenuated human IgG1 Fc domain	CUE-101 displays selective binding, activation, and expansion of HPV16 $E7_{11-20}$-specific $CD8^+$T cells from PBMCs relative to nontarget cells. Anticancer efficacy and immunologic memory were demonstrated in TC-1 tumor-bearing mice treated with a murine surrogate (mCUE-101)	Phase I recruiting	[119]
NCT02866006	Metastatic or recurrent HPV16 or 18-positive cervical cancer	BVAC-C	BVAC-C is an immunotherapeutic vaccine based on B cells and monocytes transfected with recombinant human papillomavirus (HPV) 16/18 E6/E7 gene and loaded with alpha-galactosyl ceramide	The ORR was 11%, and disease stabilization was observed in five patients. The median progression-free survival (PFS) in the total population of patients was 6.8 months, and the PFS rate at 6 months was 56%	Phase I completed	[120]
NCT02865135	HPV 16^+oropharyngeal, cervical and anal cancer	DPX-E7	A DepoVax™ (DPX)-based vaccine containing fusion peptide and adjuvant. The HPV16 E7 (H-2Db) peptide RAHYNIVTF49–57 (R9F) containing the CTL epitope was fused to a PADRE-containing CD4 T-helper epitope to generate the fusion peptide	The DPX-E7-vaccinated group contained the highest proportion of tumor-free C57BL/6 mice (>80%). Larger numbers of $CD8^+$-T cells were found to infiltrate tumors from DPX-E7-immunized mice than tumors from PBS control mice.	Phase I/II	[121]

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Table 2. (*Continued*)

Clinical trial No.	Cancer type	Name	Principle	Treatment effect	Status	References
NCT00257738	MAGE-A3 positive tumor and/or HPV 16 positive head and neck squamous cell carcinoma	MAGE-A3/HPV 16 Trojan peptides 0001 and 0002	Two novel Trojan peptide complexes, composed of MAGE-A3 and HPV 16 epitopes	None of the 5 patients immunized displayed a complete or partial response	Phase I completed	[122]
NCT04892043	HPV16+metastatic solid tumors	SQZ-AAC-HPV	SQZ® Activating Antigen Carriers (AACs), derived from engineered red blood cells, are designed to transport tumor-specific antigens and adjuvants to a patient's own professional antigen-presenting cells. SQZ-AAC-HPV targets E6 and E7 oncoproteins	In preclinical studies, SQZ® AACs have been shown to induce robust immune responses to CD8 T-cell infiltration and were correlated with tumor reduction	Phase I recruiting	https://sqzbiotech.com/patients-2/
NCT05357898	HPV16+metastatic solid tumors	SQZ-eAPC-HPV	Delivering five different mRNAs (targeting E6 and E7) to the patient's monocytes	In preclinical studies, SQZ® eAPCs have been shown to generate robust $CD8^+$T cell responses to multiple antigens through simultaneous expression of the antigens CD86, membrane-bound IL-2 and membrane-bound IL-12	Phase I/II recruiting	https://sqzbiotech.com/patients-2/
NCT04084951	HPV16+metastatic solid tumors	SQZ-PBMC-HPV	SQZ-PBMC-HPV is a novel cancer vaccine generated from peripheral blood mononuclear cells (PBMC) squeezed with HPV16 E6 and E7 antigens, resulting in delivery into the cytosol	Three patients (of 17 patients evaluated) displayed an increase in $CD8^+$tumor-infiltrating lymphocytes (TILs) and FoxP3+cell density	Phase I completed	[123,124]
NCT03260023	Metastatic or refractory/recurrent HPV16+cancer: cervical, vulvar, vaginal, penile and anal	TG4001 and avelumab	TG4001 is a suspension of MVATG8042 vector particles containing modified E6 and E7 genes from HPV16; avelumab is a fully human IgG1 anti-PD-L1 mAb	48% of patients were clinical responders 6 months after TG4001 treatment. Avelumab had already been approved by the FDA following clinical trials	Phase I/II recruiting	[125,126]
NCT05232851 NCT04260126	High-risk human papillomavirus-associated oropharynx cancer	PDS0101 and pembrolizumab	PDS0101 is a lipid-based vaccine containing six HLA-unrestricted epitopes against HPV16 E6 and E; pembrolizumab is a mAb with a high affinity for PD-1 for inhibition of PD-L1 binding to tumorigenic cells	PDS0101 has been shown to be safe in phase I clinical trials (NCT02065973) and has met the secondary endpoints for the regression of cervical dysplasia	Phase II recruiting	[127,128]

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Table 2. (*Continued*)

Clinical trial No.	Cancer type	Name	Principle	Treatment effect	Status	References
NCT02426892	HPV16+solid tumors	ISA101 and nivolumab	ISA101 is a synthetic long-peptide anti-HPV16 vaccine covering the complete sequence of E6 and E7; nivolumab is used for PD-1 immune checkpoint blockade	This treatment gave a promising response rate of 33% and remains promising in long-term follow-up	Phase II completed	[129,130]
NCT04646005	HPV16+cervical cancer	ISA101 and cemiplimab	Cemiplimab is an anti-PD-1 monoclonal antibody	N/A	Phase II	https://clinicaltrials.gov/study/NCT04646005
NCT04432597	HPV positive cancers	PRGN-2009 alone or in combination with M7824	PRGN-2009 is a therapeutic gorilla adenovirus vaccine containing multiple cytotoxic T cell epitopes from the viral oncoproteins HPV16/18 E6 and E7; M7842 is an anti-PD-L1/TGF-β trap fusion protein	PRGN-2009 treatment reduced tumor volume and increased $CD8^+$and $CD4^+$T cell levels in the tumor microenvironment of humanized mice bearing the human cervical tumor	Phase I/II	[131]
NCT03162224	HPV-associated recurrent/metastatic head-and-neck cancer	MEDI0457 plus durvalumab	MEDI0457 is a DNA vaccine targeting HPV16/18 E6/E7 with IL12 adjuvant	The objective response rate was 27.6%. However, 28 (80.0%) patients had treatment-related adverse events	Phase I/II completed	[132]
NCT03444376	HPV16- or HPV18-positive cervical cancer	GX-188E and pembrolizumab	GX-188E (tirvalimogene teraplasmid) is a therapeutic HPV DNA vaccine that encodes HPV-16 and HPV-18 E6 and E7	At 24 weeks, 11 (42%) of 26 patients achieved an overall response; four (15%) had a complete response and seven (27%) had a partial response	Phase II ongoing	[133]
NCT05286060	HPV16- and/or HPV18-positive head-and-neck cancer	GX-188E, GX-I7 and pembrolizumab	GX-I7 (hyleukin-7) is a long-acting form of recombinant human IL-7 fused to a hybrid Fc	GX-I7 promotes the proliferation, persistence and cytotoxicity of human CAR T cells in xenogeneic mouse models	Phase II recruiting	[134]
NCT04405349	HPV16- positive cervical cancer	VB10.16 and atezolizumab	VB10.16 targets the cancer-specific full-length HPV16 E7 and E6 antigens	A phase I/IIa trial of the use of VB10.16 for high-grade cervical intraepithelial neoplasia has been completed. In this phase II trial, VB10.16 plus atezolizumab gave an ORR of 19% with a median response duration of 17.1 months and a disease control rate of 60%	Phase II ongoing	[135]
NCT05799144	PD-L1- and HPV- positive oropharyngeal cancer	pBI-11 and TA-HPV	pBI-11 is a circular DNA vaccine that expresses HPV16 E6/E7 and HPV18 E6/E7. TA-HPV is a vaccinia virus vaccine that expresses HPV16 and HPV18 E6-E7 fusion proteins	Priming with pBI-11 DNA followed by boosting with TA-HPV in conjunction with anti-PD-1 can generate significant antitumor effects	Phase II recruiting	[136]

Abbreviation: ORR: Overall response rate

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6. Conclusion

Due to the low vaccination rates in developing countries and the considerable number of individuals still susceptible to HR-HPV infections, there is a pressing need for studies investigating the pathogenic mechanisms of HPV infection. Exploring clinical treatments targeting the oncogenic mechanisms of HPV should thus be a high priority in research. The integration of the HPV genome into the host DNA is a key step in the induction of carcinogenesis by HPV. In this review, we briefly explore the potential mechanisms of HPV integration and its effects on the host genome. The emergence of novel sequencing methods has provided robust technical support for identifying integration sites. With the rapid development of third-generation sequencing, the complex effects of HPV integration on host genes and chromosomes can increasingly be elucidated in greater detail. However, further studies involving larger cohorts are required to validate the accuracy of these novel methods. Drugs targeting the genes affected by integration could be employed in the treatment of HPV-related tumors within the framework of personalized medicine. Identifying new tumorigenic genes through integration analyses, along with assessing the levels of the molecules they encode, holds promise as effective biomarkers for diagnostic purposes, tumor risk assessment, and prognostic surveillance.

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Conflict of interest

The authors declare no conflicts of interest.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Epigenetic perspective on atherosclerotic cardiovascular diseases: The holistic principle of systems biology and epigenetic reasoning

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Abstract

Atherosclerosis and coronary artery disease are the main causes of impairment and cardiac death, placing a significant burden on our health-care system. This review focuses on elucidating the involvement of various epigenetic mechanisms in the genesis and progression of cardiovascular diseases (CVDs), particularly chronic CVDs. The deregulation of epigenetic mechanisms plays a crucial role in the progression of CVDs, prompting exploration into novel preventive approaches. Advancements in molecular procedures, network-based approaches, and data analysis have identified new targets in CVDs, permitting the utilization of individualized epigenetic factors for personalized diagnosis and treatment. While promising for improving diagnostic and prognostic assessments, the clinical implementation of epigenetic biomarkers lags behind. Multicenter clinical documentation in a large sample population is crucial to confidently ascertain the clinical utility of specific epigenetic biomarkers. Of particular interest is the interplay between epigenetics and the conflict between the gene-based reductionist theory and the holistic principle of systems biology (SB). The holistic principle analyzes the structural organization and regulation of biological networks, influencing the genesis and progression of complex cardiac diseases like CVDs. This review emphasizes the complexity of CVDs, elucidates the interrelationship between disease networks and epigenetic mechanisms, and highlights the importance of the holistic principle of SB, coupled with artificial intelligence, in clarifying this interrelationship. The constant and uninterrupted epigenetic impact holds immense potential for advancing our understanding of disease progression and treatment across cells and tissues. Despite these advancements, the full integration of the epigenetic impact into medical practice remains incomplete, with limited utilization in clinical applications. Nevertheless, it is likely that in the near future, the epigenetic regulation of gene expression, with its lifelong and extended effects on health, will become an integral part of everyday clinical practice.

Keywords: Epigenetics; Holistic thinking; Systems biology; Cardiovascular disease; Atherosclerosis; Artificial intelligence

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1. Introduction

Twenty years ago, it was acknowledged that the human genome contained 20,000 protein-coding genes, which represent a mere 2% of our DNA. The remaining 98%, previously dismissed as junk or dark genome, is now recognized for its critical role.

This dark genome serves as a fundamental regulator in the decoding process, or expression, of protein-making genes. Moreover, it exerts control over "how our genes behave in response to all the environmental pressures our bodies face throughout our lives, ranging from diet to stress, pollution, exercise, and how much we sleep, a field known as epigenetics[1]." Atherosclerosis is characterized as a chronic inflammatory disease involving large to medium-sized coronary or peripheral arteries, marked by progressive and complex arterial changes. Clinically, associated atherosclerotic plaques emerge, leading to cardiovascular diseases (CVDs). In this review, the terms coronary artery disease (CAD) and chronic progressive CVD are used interchangeably. Epigenetic research has expanded into the cardiovascular domain, aiming to reveal complex network interconnections between epigenetic processes and the genesis and progression of CVDs. The subjects of acute myocardial infarction (AMI) and heart failure (HF) are sporadically referenced in this context.

Clinically, relevant arterial plaques entail the interaction of different cells that give rise to infiltration of immune cells, dysfunction in endothelial cells, proliferation of vascular smooth muscle cells (VSMCs), activation of monocytes and macrophages, and the formation of foam cells. Additional significant factors contributing to the development of cardiovascular events include oxidative stress and certain coronary hemodynamic parameters (coronary vessel wall thickening and wall shear stress). Non-coding RNAs (ncRNAs), encompassing short non-coding RNAs (microRNAs or miRNAs), long non-coding RNAs (lncRNAs), and circular non-coding RNAs (circRNAs) have been identified as crucial mechanistic regulators of messenger RNA (mRNA) and protein expression, potentially contributing to atherosclerosis[2]. The term "epigenetics" is interpreted as the inquiry of inheritable and transient changes in gene expressions and phenotypes without altering the normal DNA sequence. It encompasses genomic mechanisms regulating gene expression and is associated with chemical modifications of DNA or responses to environmental and behavioral changes. The involved genomic mechanisms comprise DNA methylation, histone modifications, and RNA-based mechanisms[3]. According to Alghamdi *et al.*[4], epigenetics is the exploration of inheritable changes in gene expressions and phenotypes primarily resulting from alterations in chromatin or its packaging, thereby changing DNA accessibility.

Comprehending regulatory elements, such as enhancers and promoters, is crucial in deciphering cell type-specific gene expression patterns that instigate diseases in complex tissues[5]. The epigenome comprises diverse biological substances and proteins capable of binding to DNA, regulating functions such as activating or suppressing genes and directing the synthesis of novel proteins. Environmental and behavioral stimuli can leverage epigenetic regulatory processes in gene expression, impacting certain disorders, and increasing their risk by altering primary genetic predisposition[6]. In CVDs, numerous epigenetic modifications are implicated, yet despite advancements in epigenetic research over the past 50 years, clinical applications remain unsatisfactory[7]. The connection between external risk factors, such as environmental elements and behavioral manners, and sequence-independent heritable DNA changes is significant. This connection induces significant changes in cellular differentiation and function, influencing the health and adaptability of the organism[8].

The role of genetics is evident in certain cardiomyopathies, particularly in cases related to mutations in specific genes linked to the sarcomere. However, in complex CVDs, genetic changes are more intricate and challenging to comprehend, often perpetuating from one generation of cells to the next[9]. Epigenetic mechanisms and factors collectively establish a mechanistic link between environmental exposures transmitted through epigenetic mechanisms and gene expression profiles, influencing the development and progression of CVDs. Consequently, the epigenome presents novel categories of therapeutic targets[9,10]. Perrino *et al.*[11], in a "Position Paper," propose the integration of epigenomic and transcriptomic data as an advantageous procedure to identify crucial disease networks in patients with CVDs. They underline the potentials and limitations of these procedures and endorse innovative diagnostic or therapeutic targets, particularly for acute ischemia or reperfusion injury and ischemic HF in the post-genomic stage. At present, routine clinical practice predominantly employs a reductionist approach to diagnosis and treatment[7,12]. The holistic methodology of SB, incorporated into everyday clinical practice, is envisioned as a more relevant and personalized diagnostic and therapeutic approach, considering presumed genes and molecular networks underlying CVDs[13].

In this review, we underscore several key facets: (i) the epigenetic regulatory mechanisms linked to cardiovascular risk factors and their effect on our understanding of CVDs, (ii) the potential for epigenetic changes to affect the clinical cardiovascular phenotype, thereby expanding our opportunities for novel diagnostic and therapeutic approaches, (iii) the broader implications of epigenetics in diverse research domains such as the environment and social sciences, (iv) the involvement of epigenetics in the ongoing conflict between the gene-based reductionist

theory and the holistic principle of SB, which analyzes the structural organization and regulation of biological networks, and (v) the promising potential of artificial intelligence (AI) to interconnect epigenetic disease-related networks with CVD networks, encompassing clinical progression, prediction, and prevention (Figure 1).

2. Epigenetics, ethics, and the holistic principle of systems biology

Systems biology investigates the effects of biological networks, pathways, and molecular spatial constructions in both single-cellular and multicellular systems, addressing complex diseases and personalized medicine. This approach has demonstrated its significance across various scientific domains, including philosophy, politics, environment, economy, social sciences, and humanities. In addition, the implication of epigenetics in the conflict between the gene-based reductionist theory and the holistic principle of SB significantly influences the genesis and progression of complex CVDs.

2.1. The emergence of epigenetics

Originally coined by Conrad Waddington in 1942, the term "epigenetics" was intended to elucidate the causal mechanisms through which genes give rise to phenotypic effects[14]. Epigenetics, as defined by Russo *et al.*[15], encompasses heritable changes not explained by the DNA sequences. This notion was revised with advancements in molecular research technologies and the recognition of variations in gene expression influenced by environmental factors[16]. In a historical overview, Holliday[17] suggested that the human epigenome proposal would unravel the pattern of DNA methylation in different tissues, providing insights into the regulation of gene expression at the DNA or chromatin level or both. The Oxford conference "The Many Faces of Epigenetics: Multidisciplinary Perspectives 'over' Genetics," held in December 2017, underscored the transdisciplinary nature of epigenetics, situated at the crossroads of different fields of study. Epigenetics intersects Chemistry, Biology, Medicine, Philosophy of Science, History, and Social and Political Sciences, offering the opportunity to explore molecular or medical subjects from diverse perspectives. Trans-disciplinary studies in epigenetics can address specific issues requiring collaboration among experts from different scientific areas[18]. Nicolosi and Ruivenkamp[19] argue that the epigenetic interpretation of life is grounded in the concept of the remarkable plasticity of organisms, emphasizing the significance of the synergy between organisms and their environments, with implications for social sciences. Cavalli and Heard[20] analyze the connection between epigenetics and DNA sequence differences, exploring the entanglement of epigenetics in cellular memory and plasticity, including intergenerational and transgenerational epigenetic inheritance. Furthermore, the development of drugs related to epigenetics holds promising prospects, potentially offering increased specificity, reduced side effects, and lower drug resistance for patients with CVDs[21].

2.2. Ethical and social implications

Chiapperino[22], in an invited review published in the British Medical Bulletin (2018), argues that the field of epigenetics within the biosciences has equally inspired biomedical, social sciences, and humanities. Epigenetics does not introduce new ethical issues different from those related

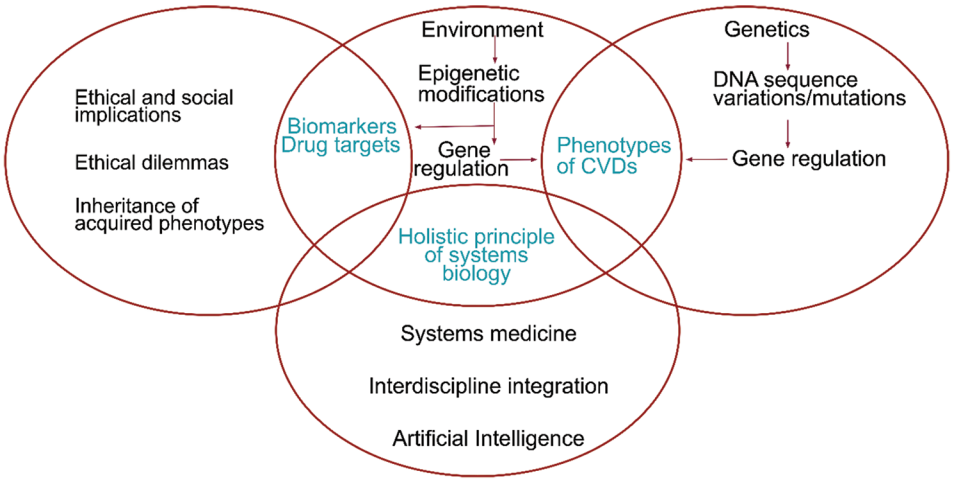

Figure 1. Complexities of CVDs: The implementation of the holistic principle of systems biology on epigenetic modifications to understand the pathophysiology and clinical course of CVD phenotypes.
Abbreviations: CVD: Cardiovascular diseases; DNA: Deoxyribonucleic acid.

to genetics. However, it does raise questions regarding the privacy and health status of subsequent generations, as well as issues related to health justice, equality of opportunities, and ethical questions. The impact of epigenetic sciences extends to addressing fundamental and contentious issues in ontogenesis, encompassing the malleability of our own bodies, receptiveness to environmental and social conditions throughout life, and the importance of these processes in adaptation, inheritance, and evolution[22,23]. Recognizing the societal importance of interdisciplinary integration, efforts are being made to facilitate the reuse of clinical data for the benefit of patients. This collaborative approach poses ethical challenges related to patient autonomy and privacy, but it also presents an opportunity for progress by borrowing concepts from one field to another while maintaining patient confidentiality in view of complex ethical dilemmas. Ethical implications in epigenetics become particularly pertinent when patients undergo screening for diseases connected to lifestyle and environmental exposures, potentially leading to issues of discrimination and stigmatization[24]. Dupras *et al.*[25] used the term epigenetics to outline the relationship between toxic exposures and epigenetic modifications, epigenetic variants and diseases, and the inheritance of acquired epigenetic traits. In addition, ethical issues emerge in the research and development of epigenomic technologies, prompting questions about guidance for researchers in the field[26]. Contrary to social epigenetics, Deichmann[27] disputes and emphasizes the focus on the environment and Lamarckian inheritance in social sciences, accrediting epigenetics with epistemic prestige.

2.3. The holistic principle of systems biology

Conventional healthcare systems traditionally employ a reductionist approach, wherein medical problems are simplified to isolated organ problems or biochemical faults. The term "disease" itself indicates that specific molecular systems or organs are malfunctioning, thereby impacting human health[28-30]. Nevertheless, when dealing with complex atherosclerotic processes and CVDs, it becomes evident that these conditions are not attributable to single-gene diseases. Instead, a holistic approach is imperative for interpreting the progression from the atherosclerotic plaque to the clinical phenotypes of CVDs. These ailments are inherently multifaceted and progressive, defying explanations through the lens of single-nucleotide polymorphisms or genome-wide case–control association studies (GWAS)[31,32]. Systems biology embodies a basic holistic perspective, fostering an understanding of interactive and integrative disease networks that explore the entirety of clinical phenotypes and disease progression rather than focusing solely on isolated pathological components of the disease (Table 1).

Table 1. Clinical understanding of chronic cardiac diseases with reductionism and systems biology approach (Reused from Louridas and Lourida[147])

Medical applications	Reductionism's objectives	Systems biology holistic strategy
Clinical focus	Isolated clinical parameters	Interactions between components, such as molecules, networks, modules, and models (phenotypes)
Prevention	Isolated culprit molecular and environmental parameters	As an entity, the whole range of culpable variables
Diagnosis	Isolated molecules, biomarkers, signs, and symptoms	The patient as a "diseased person"
Therapy	Treating causes and symptoms	Treating the patient from a holistic perspective

The holistic principle of SB presents an active and expanding scientific field grounded in the principles of integrative computational analysis. It produces interactive pathological networks and phenotypes, offering broad applications in clinical medicine[12]. To understand the concept of the holistic principle of SB, we should briefly turn to some recent developments in biology. The Modern Synthesis (Neo-Darwinist) is founded on a reductionist approach. It posits a gene-centered theory of evolution, deconstructing biological systems into constituent parts to elucidate the chemical principles governing molecular activities[12,33]. In contrast, biological relativity (BR) is a new theory of evolution emphasizing the integration of diverse biological mechanisms and networks manifested by dynamically functional systems. BR posits the absence of an authoritative level of causation in biology[34], asserting that "life is systems within systems within systems, and there is no single point of master control," while causation remains active within networks comprising components devoid of DNA templates[35]. Genes are activated through a variety of cellular mechanisms, and dynamic functional networks interrelate with the environment[36]. Boi and Lobo[37], in their exploration of the "geometry and phenomenology of the living," argue that novel concepts are required to disentangle multifactorial genetic, epigenetic, and environmental causation, explaining the inherent complexity. SB helps to understand how a biological system reacts to alterations in the microenvironment within cellular or tissue contexts, even affecting distant organs[38]. The methodology of SB has, in a way, substituted molecular biology by shifting focus toward more extensive biological constructs such as networks and signaling information systems[39]. Systems healthcare embodies a holistic approach to health, seamlessly integrating data

ranging from molecules to phenotypes and extending to encompass societal to environmental factors[29,40,41].

The holistic principle analyzes the structural organization and regulation of networks with functional properties, which "emerge" through a self-organized procedure complying with the foundations of a hierarchical multileveled system. In the context of clinical cardiology, two interrelated holistic concepts in SB are pertinent: the emerging properties with an upward direction (with upward constraints) and the proposal of constraint-applied causation with a downward direction (downward constraints) from the phenotype to epigenetic factors. (Figure 2)[12,29,42].

The holistic principle of downward (top-down) causation within the biological ladder, characterized by an integrated systems view, stands in contrast to the reductionist approach, which conceptualizes biological reality as upward (bottom-up) causation. The holistic principle elucidates the inadequacy of relying solely on bottom-up causation in the context of multi-level biology. Integrative network analysis and constraint-based thinking emerge as important tools for gaining a more profound understanding of the intrinsic complexity of CVDs[12,43-45].

The field of developmental biology was revolutionized through the advent of "omics" technology and Clustered Regularly Interspaced Palindromic Repeats genome engineering. These innovations have propelled biology toward holism, offering a new perspective on development[46]. Despite these strides, the integration of epigenetic biomarkers into routine medical practice remains limited. Similarly, personalized medicine and precision medicine have not gained widespread recognition in clinical applications, with a prevailing tendency toward a reductionist clinical approach in the treatment of patients with complex CVDs. To advance beyond this, traditional medicine needs to advance into the era of network medicine, aligning with the precision medicine paradigm to improve our diagnostic and therapeutic capabilities (Figure 3)[47].

3. Epigenetic alterations in atherosclerosis and CVDs

Epigenetic regulatory mechanisms encompass various processes:

(i) DNA methylation.
(ii) Post-translational histone modifications on proteins: Histone modifications, while not altering the DNA nucleotide sequence, can modify its availability to the transcriptional apparatus.
(iii) Post-transcriptional regulation of gene expression by ncRNAs.

Epigenetic changes resulting from these mechanisms can lead to the silencing, downregulation, or upregulation of gene expression[48] (Figure 4).

The majority of genetic diversity associated with a disease is identified within non-coding areas of the genome, with epigenetics playing a regulatory role in a downward direction. Cellular-based regulation of gene expression during transcription and translation primarily occurs

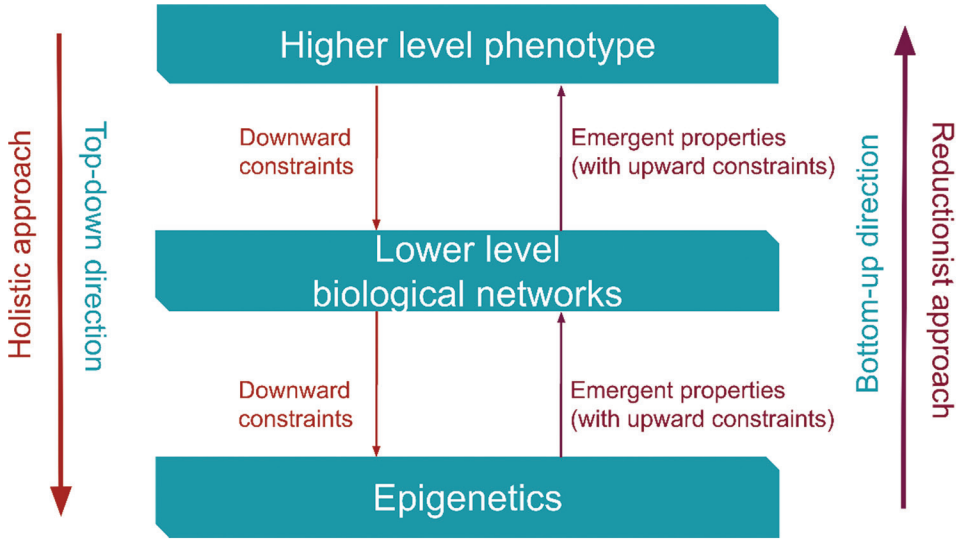

Figure 2. Constraints and emergent properties are two concepts in systems biology that explain the interrelationship and interaction between higher and lower levels of the biological ladder. Epigenetics encompass DNA methylation, histone, non-coding ribonucleic acids (ncRNAs), and environmental exposures (Adopted from Lourida and Louridas[12] with modifications).

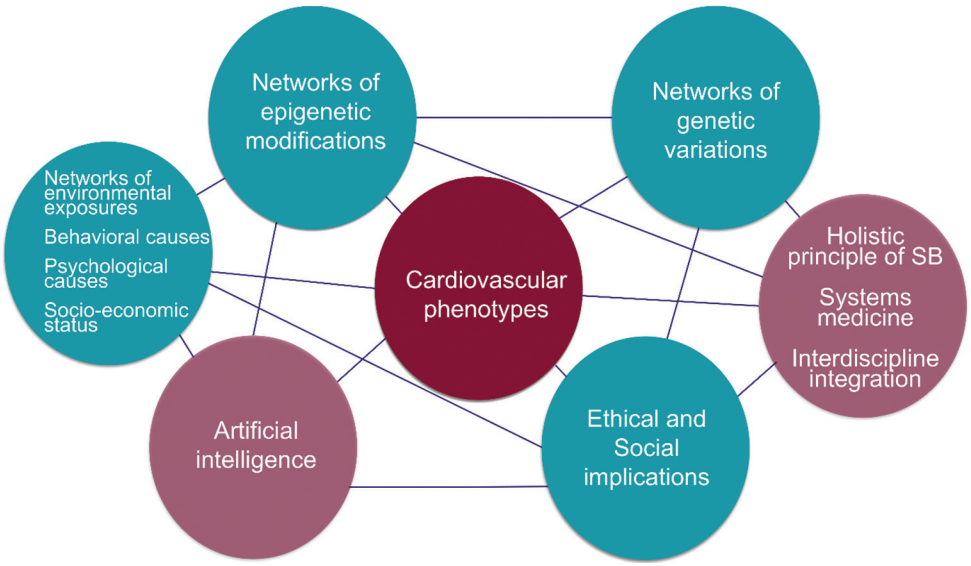

Figure 3. Cardiovascular phenotypes: The network complexity between cardiovascular phenotypes, epigenetics, and other causal factors (Adopted from Louridas and Lourida[32] with modifications).
Abbreviation: SB: Systems biology.

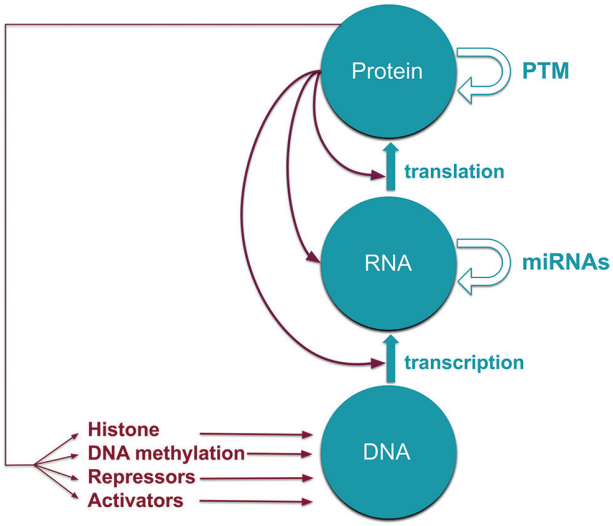

Figure 4. Epigenetic processes involve interactions among cellular molecules, indicating the transfer of information and/or the regulation of these transfer processes (Adopted from Robinson *et al.*[38] with modifications).
Abbreviations: DNA: Deoxyribonucleic acid; miRNAs: microRNAs; PTM: Post-translational modification; RNA: Ribonucleic acid.

through chromatin packaging. Chromatin, a composite system comprising DNA and DNA-binding histone proteins, orchestrates the organization of DNA packaging and its accessibility to transcription factors. Chromatin undergoes restructuring, adopting a compact condition (heterochromatin) or an open structure (euchromatin), thereby permitting the regulation of gene expression[17,49-51].

The three above-described epigenetic regulatory mechanisms play a role in the atherosclerotic process, incorporating small non-coding ribonucleic acids (sncRNAs). In addition, recent revelations underscore the potential of miRNAs and lncRNAs as biomarkers[52]. According to Xu *et al.*[53], atherosclerosis is not solely a chronic inflammatory and lipid-depository disease but is also characterized as an epigenetic disease. A growing body of evidence attests to the involvement of epigenetic modifications in the genesis and progression of atherosclerosis and plaque development. This recognition underlines the importance of epigenetic biomarkers as predictors of CVDs and emphasizes the therapeutic potential of epigenetic drugs in cardiovascular therapeutics. Notably, low-density lipoprotein (LDL) cholesterol and oxidized LDL have been proposed as stimulants that sustain an everlasting epigenetic system, remaining active even after the elimination of atherosclerotic stimuli[54].

3.1. DNA methylation

Any irregularity detected in the DNA methylation process is known to be implicated in the genesis of CVDs and can serve as a marker for evaluating CVD progression. DNA methylation, facilitated by DNA methyltransferases (DNMTs), represents an important post-translational epigenetic regulatory mechanism. While various mechanisms of methylated modification exist, the majority occur on cytosine phosphate guanine (CpG) islands in the gene promoter region, allowing for the transfer of genetic information to offspring DNA through

the regulation of DNMTs[21]. In the quest for potential biomarkers, DNA methylation microarray analyses were conducted to uncover CpG methylation profiles during the atherosclerotic process in the human aorta and in human cells[55-58]. The upregulation of DNMT1 by LDL cholesterol, leading to the methylation and repression of the Krüppel-like factor 2 gene (*KLF2*) promoter, results in endothelial dysfunction. Simultaneously, the upregulation of *DNMT3a* in human aortic endothelial cells induces methylation and repression of the Krüppel-like factor 4 gene (*KLF4*) promoter, contributing to regional atherosclerosis[59,60]. Furthermore, DNA methylation and hydroxymethylation were identified in blood mononuclear cells from elderly patients with CAD, showing a correlation with the severity of coronary atherosclerosis[61].

Zhang and Zeng[62] propose that environmental factors play a critical role in shaping the epigenetic trait of genes without altering the DNA sequence, leading to significant repercussions on cellular performance. Furthermore, they suggest that abnormal DNA methylation disrupts the transcription and expression of critical regulatory genes, resulting in the development of a proatherogenic cellular phenotype. This phenotype manifests as endothelial cell dysfunction, abnormal VSMC proliferation, extracellular matrix formation, and inflammation in CVDs.

In another study, Zhang *et al.*[63] employed bibliometric and visual methods to unravel scientific areas and trends in CVDs, aiming to anticipate the direction of future research in epigenetics. Notably, there have been limited bibliometric studies in this subject area to date. Their analysis, based on the Web of Science Core Collection, identified a total of 2,617 publications related to DNA methylation in CVDs. The United States of America, China, and England were the top three countries contributing to the field of DNA methylation.

Palou-Marquez *et al.*[64] designed a study to integrate DNA methylation and gene expression data, aiming to identify biomarkers associated with CVDs risk. They retrieved data from the Framingham Offspring Study, a cohort study with data on DNA methylation and gene expression. Four independent factors related to inflammation, endothelium homeostasis, visceral fat, cardiac remodeling, and lifestyles were identified as contributors to the determination of cardiovascular risk.

In a similar vein, Fernandez-Sanles *et al.*[65] sought to identify differentially methylated loci associated with AMI and assess their validity as predictive and causal biomarkers. They recognized 34 CpGs related to AMI, shedding light on the significance of smoking, lipid metabolism, and inflammation in the biological mechanisms related to AMI.

3.2. Histone modifications

Histone modifications constitute epigenetic regulatory mechanisms that act on histone tails through processes such as methylation, acetylation, phosphorylation, adenylation, ubiquitination, and adenosine diphosphate ribosylation[21]. These post-translational changes play a crucial role in remodeling chromatin structure and regulating gene expression by influencing chromatin accessibility, representing the most extensive group of chromatin modifications identified to date[56,66]. The regulatory impact of histone modifications extends to observed cellular functions, suggesting that histone analysis holds significant potential as the missing bridge between genomics and proteomics. Alterations in chromatin conformation can be linked to modifications in gene expression and, consequently, phenotype[67]. The reliability of histone analysis for identification and quantification is speculated to be high, paving the way for its potential incorporation as a routine method in SB. Integrating histone mass spectrometry results with genomics and proteomics datasets could enhance the comprehensiveness of SB methodologies[67].

Endothelial-mesenchymal transition (EndMT) is a cellular differentiation activity where endothelial cells undergo a change in their functional or phenotypical type, acquiring mesenchymal-like characteristics that contribute to the formation and development of atherosclerotic plaques. While numerous studies have implicated EndMT in myocardial fibrosis, myocardial hypertrophy, and hypertension, the molecular mechanisms motivating EndMT are still in the preliminary stage. Jun *et al.*[68] emphasize the role of histone modifications in EndMT in CVDs, targeting histone-modifying enzymes for CVD therapy. Lecce *et al.*[69] specifically recognize histone deacetylation, mediated by HDAC9, as a contributor to both EndMT and atherosclerosis. In mice, *Hdac9* knockout resulted in decreased EndMT and a limited plaque area. This highlights the connection of HDAC9 to vascular pathology by influencing EndMT and establishes a pathological link between EndMT, HDAC9, and atherosclerosis. Targeting HDAC9 may prove beneficial for stabilizing plaques and slowing the progression of atherosclerotic disease[69]. Conversely, HDAC3 appears to have a protective result in apolipoprotein E deficient (apoE−/−) mice. HDAC3 preserves endothelial integrity, and its insufficiency induces atherosclerosis[70]. The expression of HDACs is upregulated when the aortic VSMCs are stimulated. On the other hand, the repression of HDACs reduces aortic VSMC proliferation by altering gene expression, providing protection against atherosclerosis[71]. Greibel *et al.*[72] described an increase in histone acetylation on H3K9 and

H3K27 in VSMCs from atherosclerotic lesions, correlating with the degree of plaque damage. Furthermore, H3K9 and H3K27 methylation were markedly decreased in atherosclerotic plaques, further highlighting their association with disease severity[72]. Yang *et al.*[73] emphasize the importance of histone methylation and explore the effects of histone methyltransferases and demethylases on the pathogenesis of CVDs. They also discuss the therapeutic potential of inhibitors of histone methylation and demethylation in the context of CVDs.

3.3. Non-coding ribonucleic acids

The ncRNAs are transcribed from the genome and function at the RNA level without being translated into proteins. Representing a diverse category of molecules, ncRNAs play roles in cell proliferation, apoptosis, and metabolism[74]. Their significance, especially in relation to mRNA's fundamental role in overseeing gene functioning, has reshaped our understanding of cellular processes. Epigenetic regulation relies on the mechanisms of ncRNAs, which vary in size from very small 22-nucleotide miRNAs to transcripts exceeding 200 nucleotides (lncRNA). These molecules participate in translation and gene regulation, with established communication between ncRNAs and other epigenetic factors. Furthermore, ncRNAs serve as dominant post-transcriptional mechanistic regulators of mRNA throughout all stages of atherosclerosis[2,75,76]. The emergence of epigenetic biomarkers in CVDs has revolutionized personalized medicine, enhancing diagnostic and prognostic capabilities, and predicting the risk of future comorbidities[52]. Within atherosclerotic lesions and circulation, specific ncRNAs or clusters of ncRNAs have been identified as therapeutic targets or biomarkers for atherosclerotic plaque progression. Clinicopathological discoveries, alongside studies in animal models, have elucidated the effects of ncRNAs on the formation and progression of atherosclerosis[2]. Macvanin *et al.*[77] address the increasing prevalence of diabetes mellitus (DM) and CVDs worldwide, highlighting numerous regulatory ncRNAs associated with these conditions. NcRNAs are also explored as prognostic biomarkers and potential therapeutic elements for DM and CVDs.

The scientific expertise in the field of ncRNAs encounters certain limitations, primarily stemming from the absence of multicenter surveys with persuasive verification for clinical applicability. The possibility of diverse formations containing various ncRNAs and other biomarkers suggested that, with the help of AI, more specific diagnostic and prognostic procedures for CVDs may be developed[48]. Selecting a cluster of biomarkers for a specific CVD poses a challenge, compounded by uncertainties regarding the standardization of collected samples from pathology departments. Issues related to sample firmness, magnitude, and coherence present challenges that need to be addressed to ensure the reliability and applicability of findings[78].

3.3.1. Small noncoding ribonucleic acids (microRNAs or miRNAs)

MiRNAs constitute a group of approximately 21- to 25-nucleotide small RNAs that exert negative control over gene expression at the post-transcriptional stage, significantly enhancing our understanding of complex gene regulatory networks[79]. However, careful attention is required, as miRNAs typically target multiple mRNAs from various genes, and each gene can be targeted by multiple miRNAs. The complexity of this network demands thorough scrutiny before a specific miRNA can be considered a reliable biomarker for a disease[80]. Extensive reviews have covered the biogenesis, identification, mechanism of action, and degradation pathways of miRNAs[81,82]. While the operative number of miRNAs in humans ranges from 556 to 758, the functionally active ones expressed at adequately high levels in tissue are more limited. In the cardiovascular system, 150 miRNAs are operative, and this number is further restricted to 30–35 miRNAs in experimental *in vivo* models[83-85]. MiRNAs regulate gene expression by inhibiting translation through the repression or degradation of targeted mRNAs at the post-transcriptional level[86-88]. Dysregulated expression of miRNAs is associated with various cellular processes, including hyperlipidemia, atherosclerosis, obesity, DM, and CVDs[88]. In a study by Kong *et al.*[89] involving 326 patients with DM and 342 healthy controls in a northern Chinese Han population, the CC genotype of rs4705342 was found to increase the expression of miRNA-143, potentially acting as a risk factor for DM. However, the relationship between circulating and tissue miRNAs remains unclear, as many surveys reveal that miRNA levels in the blood may not accurately reflect alterations in tissue levels, given that miRNAs can also be produced by immune cells[90].

(a) miRNAs in atherosclerosis

MiRNAs hold promise as stable molecular biomarkers and potential drug targets, with modifications tailored to specific tissues or diseases. However, the current literature lacks extensive records on miRNA expression in human atherosclerosis, and the role of miRNAs in human atherosclerotic arteries remains largely undefined[91]. In a mouse model featuring proatherogenic apoE$^{-/-}$ mice, the presence of miRNA-217 was associated with diminished nitric oxide production and increased endothelial dysfunction, suggesting therapeutic potential

for miRNA-217 inhibitors in CVDs[92]. Atherosclerosis has been shown to decrease following the regulation of specific genes at the transcriptional or post-transcriptional level, with DNA methylation playing a role in ceasing relative gene expression[93]. Yang *et al.*[94] identified microRNA-216a as an inducer of endothelial senescence and inflammation, acting as an inhibitor of the Smad3/IκBα pathway and proposing it as a novel biomarker for elderly individuals with atherosclerotic diseases. Plasma levels of miRNA-216a were higher in older CAD patients, correlating with a 31% increased risk compared to healthy controls.

Chen *et al.*[95] found that elevated levels of circulating miRNA-17-5p could serve as a diagnostic biomarker for atherosclerosis. Li *et al.*[96] evaluated the prospect of circulating miRNAs as biomarkers for atherosclerotic plaque rupture in stable CAD patients undergoing percutaneous coronary intervention with a single stent implantation. They identified three miRNAs, miR-155-5p, miR-483-5p, and miR-451a, as potential biomarkers for the early detection of plaque rupture[96]. Mao *et al.*[97] examined genes with differential expressions in earlier and older atherosclerotic plaques. Analyzing two public datasets from the Gene Expression Omnibus (GEO) databases, they identified a total of 23 miRNAs, with miR-19A, miR-19B, miR-126, and miR-155 standing out as potential biomarkers for carotid atherosclerosis[97].

(b) miRNAs in CVDs

MiRNAs emerge as important regulators of homeostasis in various organ systems, particularly in the cardiovascular system. Beyond their involvement in the CVD progression process, such as cardiac hypertrophy and myocardial cell fibrosis, they also serve as promising therapeutic targets[98,99]. Myocardial implications in CVDs arise from disturbances in intracellular signaling across various cell types, including VSMCs, endothelial cells, cardiomyocytes, and fibroblasts. Disturbances in intracellular signaling cascades affect heterocellular communication, highlighting the importance of identifying dysregulated miRNAs under pathological conditions[100]. However, more efforts are required to recognize irregular miRNAs responsible for the clinical understanding of atherosclerosis, cardiac hypertrophy, and complex CVDs[100]. MiRNAs possess the unique ability to simultaneously regulate multiple elements within relevant pathways, making them potential biomarkers and therapeutic targets[101,102]. Laggerbauer and Engelhardt[83] reported on the advances in the identification and characterization of miRNAs as therapeutic targets in the cardiovascular system, discussing challenges and prospects for clinical translation and application. The role of miRNAs in CVDs operates within a realm with limitations of conventional pharmacotherapy, and miRNA-based drugs have progressed significantly into preclinical and clinical testing. While there is insufficient appreciation for the role of miRNAs in the pathophysiology of CVDs, a substantial number of deregulated or disease-modified miRNAs exist, raising the question of whether the deregulation of a miRNA causes disease or merely indicates it[83]. Despite GWAS identifying polymorphisms in miRNA biogenesis factors, miRNA genes, and 3' untranslated region variants (3'UTRs) strongly associated with human traits and diseases, pathophysiological changes have been determined only in a few cases[103]. In a recent review, Maries *et al.*[104] summarize the current updates on the molecular mechanisms of over 30 miRNAs involved in post-myocardial infarction (MI) replacement fibrosis leading to left ventricular remodeling and HF.

Extracellular vesicles (microparticles), produced by human coronary artery smooth muscle cells, carry a variety of miRNAs, such as miR-21-5p, miR-143-3p, miR-145-5p, miR-221-3p, and miR-222-3p, with some of these miRNAs originating from atherosclerotic plaques[105]. Exosomes, small extracellular vesicles that transport regulatory miRNAs, mRNAs, and lncRNAs, function as intercellular messengers and information transmitters. Vaskova *et al.*[106] examined the effects on exosome production employing a chronic rodent myocardial injury model and confirmed that miR-181a antagomiR has a beneficial effect on cardiac function. Importantly, rats treated with miR-181a antagomiR displayed a noticeable recovery in left-ventricular function and cardiac remodeling. Consequently, miR-181a could serve as a valuable early biomarker of myocardial ischemia, and its downregulation by a specific antagomiR holds potential for therapeutic implementations[106]. MiR-144 has demonstrated protective effects against detrimental post-MI remodeling in both ischemia and reperfusion and non-reperfused MI mouse models[107].

In an MI mouse model with a permanently ligated left anterior descending (LAD), miR-19a/19b prevented post-infarction HF. Intracardiac injection of miR-19a or miR-19b mimics reduced the infarct site, preserved cardiac function in the post-infarct period, and increased survival[108].

In a porcine LAD occlusion MI model, the delivery of miR-199a into the left ventricular led to significant cardiac function recovery, decreased infarct size, and reduced cardiac fibrosis. Nevertheless, long-term expression of miR-199a resulted in sudden cardiac death for the majority of treated animals. This highlights the need for a more precise and constrained dosing and delivery strategy for any miRNA-based substances in clinical settings[109]. The prospect of therapy for presently incurable diseases

through the use of precision genetic or medical techniques is anticipated in the near future. Delivery of therapeutic molecules in now inaccessible target tissues, such as the cardiac infarct site, will soon be plausible. The main barrier restraining the clinical implementation of miRNA-based therapeutics is the focused, selected delivery to the infarct site[110,111].

Hong *et al.*[112] proposed, for better miRNA delivery, an anti-coagulative nanocomplex to carry and deliver a miR-1 inhibitor capable of reducing microthrombus formation and microvascular obstruction and inhibiting blood-coagulation factor Xa[112]. Bejerano *et al.*[113] introduced a nanoparticle-based delivery of a miR-21 mimic to cardiac macrophages at the infarct site in a mouse ligation model as a new therapeutic strategy. Feng and Tsao[114] expressed their apprehension about the potential dangers of the systemic effects of its miRNA mimics and suggested that any miRNA-based substances are better used locally rather than systemically. Bielska *et al.*[115] attempted to uncover circulating miRNAs as potential biomarkers for the early identification of CAD risk in DM patients and discovered six miRNAs (miR-615-3p, miR-3147, miR-1224-5p, miR-5196-3p, miR-6732-3p, and miR-548b-3p) that were overexpressed in the serum of type 2 DM patients with CAD.

A substantial number of descriptive publications on miRNAs await, pending critical evaluation for the identification and validation of the discovered miRNAs. While thousands of papers are published annually linking miRNAs to different genes and human diseases, only a limited number delve into the molecular mechanisms of miRNAs. There is a pressing need for a greater emphasis on understanding the mechanisms underlying miRNAs, and researchers seeking associations of specific miRNAs with biological phenotypes should shoulder a heightened responsibility[116].

3.3.2. Long non-coding ribonucleic acids

LncRNAs regulate gene expression by altering chromatin structure and DNA accessibility; moreover, they enhance selectivity and control numerous cellular processes such as DNA methylation and histone modification[117,118].

Belonging to a category of RNAs produced by RNA polymerase II, lncRNAs play a key role in cell processes, including transcription and regulation of gene expression. LncRNAs exhibit essential regulatory functions, interacting with proteins, other RNAs, and DNA. They also play a role in regulating biological processes related to vascular tissue homeostasis, atherosclerotic lesion formation, and plaque destabilization[119].

Thousands of lncRNAs need to be functionally categorized, considering the abundance of genomic data on lncRNAs, including sequences, transcriptome data, and interactions with connected proteins or genes. It is important to integrate data from various origins for the numerous lncRNAs to specify functions and regulatory mechanisms. A large number of lncRNAs have been identified as significant regulators of cardiac development and aging, yet their pluripotent function in cardiac development and disease, along with their diagnostic, prognostic, and therapeutic utility, remains to be determined[120]. Rezaee *et al.*[121] outline the potential of various lncRNAs to serve as diagnostic, prognostic, and therapeutic biomarkers in HF. In HF, various molecular mechanisms are markedly deregulated by divergent lncRNAs. LncRNAs can influence distinct signaling pathways by targeting molecules or cellular mechanisms, and changes in their expression are observed in diverse types of CVDs and HF, supporting the notion that they play an important in the occurrence and progression of heart diseases[121].

New biomarkers are crucial for improving early detection and predicting the progression of AMI. Wang *et al.*[122] evaluated the potential of the lncRNA GAS6-AS1 in distinguishing AMI patients and predicting outcomes. The discriminative ability of GAS6-AS1 in identifying AMI patients was evaluated using Kaplan–Meier and Cox analysis and its association with critical patient characteristics was assessed through Spearman's correlation analysis. The study concluded that decreased GAS6-AS1 expression could effectively distinguish AMI patients from healthy controls[122].

VSMCs constitute a significant cell type within atherosclerotic plaques, and their phenotypic transition gives rise to diverse cell categories within the plaques. In CVDs, the rapid proliferation and migration of VSMCs in the arterial wall contribute to the abnormal structure and function of the arterial intima-middle layer. Shi *et al.*[123] identified 4579 known and 13,655 *de novo* lncRNAs in human coronary artery VSMCs, highlighting the crucial role of lncRNAs in the phenotypic transition of smooth muscle cells and human atherosclerotic disease. LncRNAs act as upstream regulators of cellular processes associated with atherosclerosis, pulmonary hypertension, and aneurysms – three lethal CVDs[124]. In general, miRNAs and lncRNAs play essential roles in maintaining physiological homeostasis by regulating the expression of various genes. They also attract scientific interest as potential biomarkers for diagnosis and therapeutic targets in DM and CVDs[125,126].

3.3.3. Circular non-coding ribonucleic acids

CircRNAs exert their regulatory functions through specific mechanisms: (i) acting as miRNA sponges, modulating

mRNA translation by interacting with miRNA, (ii) serving as a protein scaffold; circRNAs bind to RNA-binding proteins to regulate their function and transport, (iii) potentially functioning as a molecule for transcriptional regulation, and (iv) serving as a template for protein synthesis, engaging in protein translation[21]. CircRNAs exhibit a multitude of regulatory functions associated with the pathogenesis of cardiac remodeling[127]. With the success of high-throughput sequencing technology, it has been discovered that circRNAs exhibit stable cell-type-specific or tissue-specific expression and participation in various processes, including the risk of atherosclerotic vascular disease[128]. CircRNAs have the potential to be biomarkers for CVDs, cancers, and autoimmune diseases. Recently, there has been speculation about the translational potential of utilizing human blood circRNAs as liquid biopsy biomarkers for diagnosis and prognosis[129,130]. Extracellular vesicles (exosomes, microvesicles, and apoptotic bodies) serve as information carriers that regulate intracellular interactions, with their load (circRNAs) being engaged in atherosclerosis. Current data emphasize the importance of extracellular vesicle-derived circRNAs (EV-circRNAs) in the initiation and progress of atherosclerosis and their potential use as diagnostic biomarkers or therapeutic strategies[131]. Although several software platforms have been developed for precise identification, functional prediction, and validation of circRNAs, limited cardiovascular circRNA studies have used these scientific tools[132]. Nevertheless, the importance of circRNAs as regulatory mechanisms in cardiovascular physiology and pathology is recognized, with emphasis on the unresolved challenges associated with circRNAs in the research and treatment of CVDs[133]. CircRNAs are linked with various CVDs and accepted as intracellular effector molecules in various cardiovascular activities or as cardiovascular biomarkers, with recognized interactions with DNA, RNA, and proteins[134]. Dodbele *et al.*[135] describe the development of new experimental procedures, particularly methods for estimating or regulating circRNA expression levels. However, each of these approaches has inherent limitations and potential pitfalls that necessitate careful consideration during experiment design and result interpretation. Dodbele *et al.* provide guidelines for the reliable identification, validation, and functional characterization of circRNAs to enhance clarity in the field[135]. Exogenously manufactured circRNAs *in vitro* can be integrated into cells as therapeutic molecules, either replicating endogenous circRNA or designed artificially to modulate gene expression networks *in vivo*[136]. At present, it is understood that the modulation of circRNA levels can give rise to a diversity of molecular and physiological phenotypes, impacting the nervous system, innate immunity, miRNAs, and numerous disease-relevant pathways[137].

4. Epigenetic changes following environmental exposures and lifestyles

Epigenetics is a highly rewarding research area that integrates epigenetic mechanisms with environmental, nutritional inputs, and lifestyle. Consequently, epigenetic processes, beyond serving as regulators of DNA expression, are intricately linked to environmental changes that influence the genesis and progression of CVDs (Figure 5).

This association has an impact on personal features and qualities, but it also affects the genes of our descendants. Environmental factors and personal lifestyles contribute to disease vulnerability, but the connection between the above independent exterior risk factors and our genetic mechanisms has been vague. The influence of multiple and diverse epigenetic processes in CVDs is presently under investigation, hoping to untangle novel biomarkers and clinical approaches for precision medicine[8]. Furthermore, the exact involvement of epigenetic factors during the development and clinical progression of CVDs is not well understood[138]. Restricted socioeconomic status and economic opportunities, psychosocial tensions, and inadequate education have emerged as social disadvantages and CVDs risk factors. The epigenetic patterns and modifications are represented in cellular memory, could be reversible, differ between cell types, and promote disease predisposition by inducing prolonged changes in gene transcription[139]. Complex CVDs have a genetic framework, but the eventual phenotypic after-effect relies on the patient's environment and lifestyle; besides, in patients with complex diseases, genomic studies have as yet verified only a small percentage of the risk to be inherited[140]. The absent heritability can partly be interpreted by the presence of epigenetic variation. Epigenetic mechanisms are viewed as important mediating processes between genotype and phenotype variability extending at all stages of CVDs development and progression that unlock opportunities for innovative preventive, diagnostic, and therapeutic strategies. This area of research is largely unexplored and may assist in a more constructive conception of molecular mechanisms underlying cardiovascular epigenetics and diseases[141].

Münzel *et al.*[138] state that globally, CVDs patients represent a significant fraction of non-communicable diseases (NCDs). Seventy percentages of the yearly global deaths can be attributed to NCDs, with most deaths induced by CVDs; the risk of NCDs is firmly connected to environmental stressors and genetic predisposition[138]. The American Heart Association (AHA) determined

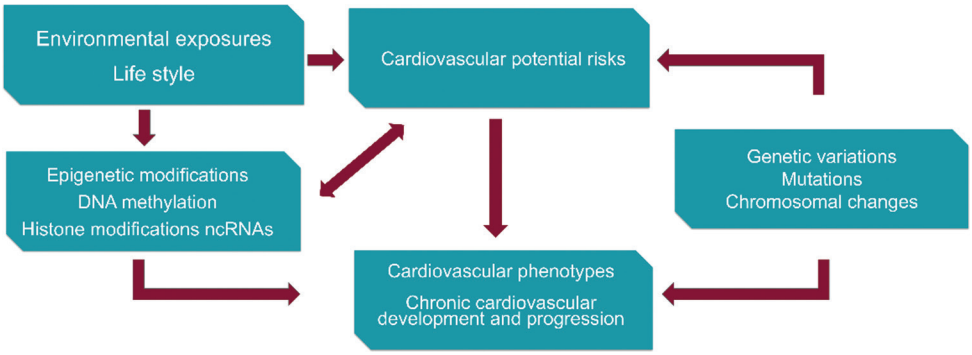

Figure 5. The effect of epigenetics on cardiovascular diseases.
Abbreviation: ncRNAs: Non-coding RNAs.

cardiovascular health (CVH) by applying the Life's Simple 7 metrics. There are four health elements and three health behaviors of lifestyle[142,143]. Joyce *et al.*[143] provided evidence that GrimAge acceleration (GrimAA), a measure of epigenetic aging, maybe a practical biomarker of CVD risk; this gives a biological understanding of the epigenetic mechanisms associated with age-related CVH decline and CVDs.

5. Systems medicine and interdisciplinary integration

The epigenetic impact is a constant and uninterrupted process with broad cell and tissue applications, increasing our understanding of disease progression and treatment. At the moment, it is not fully integrated into medical practice or extensively used in clinical applications. Epigenetic data are increasing due to the progress in high-throughput sequencing and microarray technologies. The question now is how to enlarge and analyze the data findings and identify and interpret their functional repercussions in normal development and disease[56]. The extensive parallel sequencing technologies merged with analytical molecular approaches and computational techniques have permitted clinicians and researchers to realize chronic mechanisms involved in the pathology of CVDs. As a consequence of the absence of suitable and effective computational methods, most studies concentrate on a single epigenetic element in isolation despite the fact that numerous connections from multiple constituents and genotypes are taking place *in vivo*[144]. An effective data informative statement is required for data standardization to enhance the reproduction of epigenetic discoveries[145]. A great number of epigenetic data are assembled in the domain of CVDs, but comprehension of the basic mechanistic features of CVDs remains unrevealed; a new friendly strategy and extensive information processing to elucidate disease pathophysiology is needed.

Alexandar *et al.*[146] have established a literature-based database, CardioGenBase, collecting gene-disease association from PubMed and MEDLINE, and containing approximately 1500 CVD genes from around 24,000 research articles. CardioGenBase is an indispensable online resource to uphold genome-wide analysis of genetic, epigenetic, and pharmacological studies[146].

In addition to data retrieval and manipulation, computational analysis is crucial to accurately approaching unprocessed information material and overcoming the complex fragmentation of data required. The real constraint is not the availability of data but the limited processing power, which is the critical technology needed. CVDs should be explored through different levels or sections of information, from genetic and molecular realms to clinical phenotypes, unraveling and connecting the available biological networks in pursuit of defining clinical complexity. Thus, the holistic multiscale and integrative approach of SB will increase the prospects for true systems medicine. Regardless of important breakthroughs in clinical medicine through the reductionist methodology, there are still unanswered questions and limitations for common complex diseases[147].

Ahn *et al.*[148] argue that reductionism has limitations and a different explanation is needed. The systems perspective respects the holistic and compounded attributes, solving the problem through the use of computational and mathematical tools. In another paper, the same authors claim that reductionism splits up the problem into its parts, missing important information about the whole, and disregards interactions between elements[149]. Systems medicine looks beyond linear relationships and isolated parameters, using multiple parameters from multiple time points and spatial conditions to accomplish a holistic perspective of an individual[147]. Ung *et al.*[150] argue that it remains ambiguous how epigenetic regulations consensually guide the advancement of biological memories

at the whole-body status. They propose the Manifold Epigenetic Model (MEMo) as a conceptual structure to interpret epigenetic memory emergence and consider strategies to exploit body-wide memory. The emerging field of regenerative medicine is based on the epigenetic memory extending in tissue engineering, the development of biomaterials, medical devices, and artificial organs, while cellular therapies are promising for the treatment of CVDs, diabetes, corneal blindness, and cystic fibrosis[150]. HF is also a complex clinical complication, the outcome of many different CVDs affecting the myocardium and eventually ending up with a common clinical picture. Pattini *et al.*[151] analyze the different periods of HF deterioration through the multistage approach of systems medicine. Furthermore, pursuing deterioration from one stage to another, they explore how the SB perspective and functional genomics transform the clinical approach toward diagnosis and treatment.

Green[152] argues that the diversification of models and their respectively dissimilar epistemic objectives are significant for emerging intelligible scientific theories. However, more expertise is required to understand how the synergy of various epistemic areas, such as SB, can give rise to and sustain new entities in science. Green and Andersen[153] debate that scientific co-operation between the two fields of research, epigenetics (experiments) and SB (theoretical modeling), is needed for the productive implementation of SB holistic thinking in epigenetics research. It should overcome the impediment that exists between SB and epigenetics scientists due to information boundaries and segregated research. Presenting and elucidating the disciplinary experience for the different views can benefit interdisciplinary cooperation in science[153].

5.1. Artificial intelligence and epigenetics

Artificial intelligence, developed at the intersection of technology and medicine, has swiftly integrated into medicine through digital health applications. Its goal is to make medicine more precise and error-free (Figure 6).

Thus, the rapid integration of AI into medicine increases the prospect of enhancing clinical outcomes and transforming healthcare practices. AI not only improves the quality of life and home medical care but also elevates daily clinical cardiology practices, enhancing medical or clinical information from cardiac imaging to informed clinical decisions. The capacity of AI to collect, analyze, and integrate electronic data from "omics," epigenetics, and clinical sources is significant for understanding the complexities of chronic CVDs at the individual level. Clinical AI holds the promise of improving

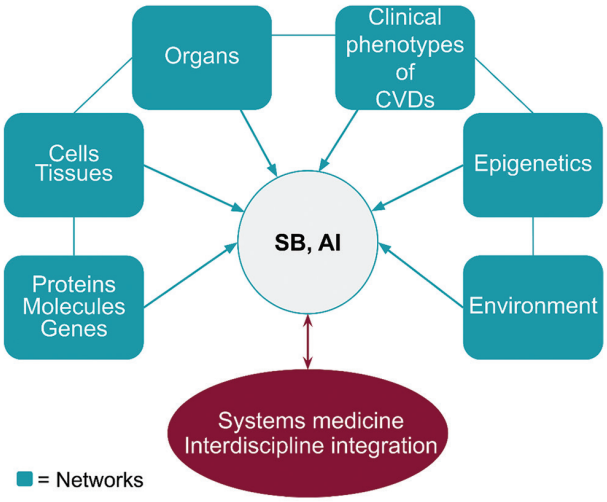

Figure 6. Schematic diagram based on the SB concept illustrating communication (links and data transmission) between subclinical and clinical stages of chronic complex CVDs. This communication concept is intricately connected to data transmission (SB holistic principle) and AI, with a specific focus on human medical data (Adopted from Lourida and Louridas[12] with modifications).
Abbreviations: AI: Artificial intelligence; CVDs: Cardiovascular diseases; SB: Systems biology.

clinical outcomes and reshaping healthcare practices by integrating clinical cardiology with information derived from epigenetic sources.

It is imperative to use AI for integrative network analysis to extract electronic health records by incorporating diverse data references, uncovering individual patient-related modes of disease progression. This incorporation of clinical data necessitates appropriate computer algorithms for risk classification and the prediction of therapeutic clinical effects and after-effects. Developing a new culture of openly sharing data making datasets and clinical study reports accessible to others is essential. Perhaps, the interrelationship and interconnection between disease networks of epigenetics with networks of clinical progression, prediction, and prevention hold the key to understanding the complex atherosclerotic CVDs[12]. The analysis and clarification of complex diseases' biological and clinical networks and the expansion of data standards could be achieved through AI. Encouraging data standards and sharing will enhance the integration of clinical and non-clinical data, leading to the development of effective AI tools[154]. AI has the potential to lead the way in subsequent medical innovation and upgrade precision medicine to differentiate patients with different phenotypic characteristics. However, its usefulness is impeded by obstacles such as an absence of adequate algorithms, a shortage of physician training, fear of over-mechanization,

and the loss of human touch[155]. Importantly, significant uncertainties still remain about how AI services will be adopted by health authorities and physicians for patients with chronic diseases such as CVDs. Moreover, the adoption of AI technology for complex interactive and integrative epigenetic networks raises questions about cost, payments, reimbursement, and the incremental value provided by AI systems. A significant issue arises concerning the use of AI to solve scientific and social problems and improve our lives. The Pew Research Center, which informs the public about the issues, attitudes, and trends shaping America and the world, published a report addressing the future of ethical AI. Based on a questionnaire addressed to experts, the report concluded the following: "The majority worries that the evolution of AI by 2030 will continue to be primarily focused on optimizing profits and social control. They also cite the difficulty of achieving consensus about ethics. Many who expect progress say it is not likely within the next decade. Still, a portion celebrates coming AI breakthroughs that will improve life."[156] Brender[157] claims that physicians should grasp how to integrate AI tools into clinical practice and be cautious of any new technology, especially one that carries existential implications. He remarks that he is "prudently optimistic about a future of improved health-care system efficiency, better patient outcomes, and reduced burnout."

6. Conclusion

Complex diseases, such as CVDs, result from a combination of genetic and environmental components. Epigenetic modifications are highly influenced by the environment, and throughout an individual's lifetime, exposure to environmental risk factors increases the occurrence of CVDs later in life. On an individual basis, everyone should be part of a program that provides improved food quality with reduced exposure to air pollution and other contaminants. Epigenetic regulation of genes associated with the atherosclerotic process is crucial. The interplay between various epigenetic factors is key to understanding the complexity of the atherosclerotic process and increases the probability of expanding our knowledge, thereby aiding in the prevention and treatment of patients with CVDs. The SB methodology of network-based processes has emerged as a dynamic tool for studying complex CVDs. Scientific cooperation is essential for the productive implementation of the SB holistic understanding in epigenetic research and clinical thinking. A suitable digital project could be undertaken to explore and interconnect epigenetic factors with biological or disease networks and clinical phenotypes. This project, run by a powerful computational program, should serve as an interdisciplinary mechanism involving the scientific community as a whole. It should bring together diverse procedures to address uncertainties that have arisen in different specialized disciplines.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Surgical outcome of open carpal tunnel release: A 2-year case series study at Zliten Teaching Hospital, Libya

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Abstract

Carpal tunnel syndrome (CTS) is the most commonly diagnosed peripheral neuropathy, with a prevalence of 1 – 16% within the general population. If left untreated, it can lead to permanent dysfunctionality and disability. This study aimed to determine the outcomes and complications associated with open carpal tunnel release surgery (OCTR), compare the presentation of CTS symptoms before and after the operation, and investigate the factors contributing to post-operative complications. To achieve these objectives, a case series design was employed, and the study was conducted at the Department of Orthopedics and Traumatology in Zliten Teaching Hospital, Libya, from January 2016 to December 2018. A total of 256 patients who had opted to undergo OCTR of the transverse carpal ligament during the preoperative stage were enrolled and prospectively followed up for 2 years. The post-operative follow-up analysis revealed a statistically significant reduction in symptoms such as dull aching discomfort in the hand and forearm, as well as paresthesia and numbness when compared to the pre-operative period (10.2% vs. 90.5%; $P < 0.0001$). However, there were no significant differences in clumsiness (18.8% vs. 25.8%; $P = 0.256$) or thenar muscle atrophy (1.6% vs. 5.0%; $P = 0.194$). The majority of the patients (90.6%) expressed high levels of satisfaction with the procedure, reporting no recurrence of symptoms. In contrast, only 9.4% of patients experienced post-operative complications. The study findings revealed no significant correlation between post-operative complications and body mass index ($P = 0.194$). Nonetheless, a statistically significant association was observed between the severity of nerve conduction and post-operative complications ($P = 0.011$). In summary, the open release of the transverse carpal ligament resulted in positive surgical outcomes.

***Keywords*:** Carpal tunnel syndrome; Libya; Open release; Outcomes; Surgery

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1. Introduction

Carpal tunnel syndrome (CTS) is defined by the American Academy of Orthopedic Surgeons (AAOS) as a "symptomatic compression neuropathy of the median nerve at the level of the wrist"[1]. This specific nerve entrapment neuropathy accounts for 90% of all neuropathies[2]. CTS stands as the most commonly diagnosed peripheral neuropathy, exhibiting a prevalence of 1 – 16% in the general population[3], with a notable predominance in females[4]. Clinically, CTS can be graded into mild, moderate, and severe forms based on the symptom's history, presentation, and severity[5]. Initial symptoms of CTS include discomfort or soreness, numbness, and paresthesia. These symptoms most frequently manifest in the thumb, index finger, middle finger, and radial half (thumb side) of the ring finger, although some variation may occur. In addition, individuals with CTS may experience soreness in the affected arm. As the condition worsens, hand weakness, impaired fine motor coordination, clumsiness, and thenar muscle atrophy may become apparent. Various factors, including fluid retention, particularly during pregnancy, can contribute to the development of CTS.[2] Patients often report a worsening of their symptoms during the night, causing sleep disturbances[6].

CTS is a frequently encountered condition resulting from the compression of the median nerve within the carpal tunnel of the wrist. However, if left untreated, it can result in long-term dysfunction and physical impairment[6]. Notably, the progression of symptoms in CTS is not always linear, and in some cases, it spontaneously resolves over time. In these circumstances, CTS may naturally resolve or respond to minimal self-care measures. In cases where symptoms persist, conservative treatments and non-surgical interventions, such as wrist splints or steroid injections, are frequently employed to manage mild-to-moderate symptoms. Surgical release (decompression) of the carpal tunnel becomes a consideration if non-surgical remedies fail to alleviate the symptoms associated with CTS[2,7].

The individualized treatment modality is customized through a comprehensive assessment of several factors, including the severity of the symptoms of the limb, the psychosocial status of the patient, and the surgeon's capacity to execute a well-outlined treatment plan with precision and safety, all aimed at achieving a successful outcome[8]. In the management of CTS, a range of surgical procedures has been described, including endoscopic carpal tunnel release (ECTR) or open carpal tunnel release (OCTR). These techniques can be further categorized as traditional open, limited open, single-portal endoscopic, and two-portal endoscopic approaches[9]. The preferred treatment method for reliably alleviating symptoms and performed in current orthopedic practice involves the decompression of the median nerve through an open release of the transverse carpal ligament[10]. In cases where surgery is indicated, it is essential to consider the potential for significant complications. The clinical symptoms that may ensue following these complications can be more severe and distressing than the patients' original complaints[1].

Irrespective of the chosen surgical technique, carpal tunnel surgery generally yields favorable results. While ECTR has been demonstrated to offer a quicker recovery time and reduced postoperative discomfort compared to OCTR, it may still result in a number of problems[11]. The standard OCTR, which involves a longitudinal skin incision directly over the transverse carpal ligament, has consistently yielded favorable outcomes[12]. However, this technique has been associated with difficult-to-treat complications, including discomfort in the scar, pain in the thenar and hypothenar (pillar) muscles, and weakness in the pinch and grasp strength[11]. A systematic review[9] was conducted to compare OCTR and ECTR methods, assessing post-surgical outcomes and complications. The study discovered that ECTR had a higher incidence of superficial palmar arch injuries and transient nerve damage between 1960 and 1990[9].

Lee and Strickland developed a limited-open technique employing specifically designed instruments known as the "Indian atome." This approach features a smaller palmar incision, measuring just 1.5 cm in length, with the aim of minimizing the trauma caused by OCTR. The authors reported achieving results similar to those performed with ECTR while experiencing fewer complications[13]. This technique combines the simplicity of open release with reduced surgical trauma, a smaller incision, and decreased postoperative morbidity. The procedure has been found to be straightforward to implement, and numerous patients have reported excellent outcomes with fewer complications[11]. Despite the advent of endoscopic release, the incidence of nerve injuries has not significantly decreased, although there has been a gradual decline in issues brought on by vascular injuries. The increased rate of transient nerve injury and the accompanying skin complications should be considered when deciding between these two widely used surgical methods[9].

Older age, female sex, and a high body mass index (BMI) have all been identified as risk factors for CTS, according to a review article[14]. High BMI was substantially associated with CTS, although not with the severity of the condition, as confirmed by a cross-sectional analytical investigation that yielded comparable results[15]. A crucial step in defining the prognostic and therapeutic measures involves

the CTS severity assessment[16]. Nerve conduction studies (NCS) serve as a useful tool for evaluating CTS, aiding in diagnosis, decision-making for the treatment plan (whether conservative or surgery), and predicting post-surgical prognostic outcomes. Notably, the previous research has linked NCS results to poor outcomes for surgical release, underscoring the significance of addressing clinical outcomes for the benefit of both patients and surgeons[17].

The objectives of this study were as follows: (i) To determine the surgical outcomes and complications following OCTR surgeries; (ii) to compare the presentation of CTS symptoms at the pre-operative stage with those observed at the post-operative follow-up; and (iii) to evaluate the factors (body mass index and nerve conduction study severity) associated with post-operative complications.

2. Materials and methods

2.1. Study setting

This study adopted a case series design and was conducted at the Department of Orthopedics and Traumatology in Zliten Teaching Hospital, Libya, spanning from January 2016 to December 2018. Patients who opted to undergo an OCTR procedure involving the transverse carpal ligament were recruited during the pre-operative stage and prospectively followed up for 2 years to assess the surgical outcomes of OCTR. The demographic information of the patients and all required data were obtained through a review of the hospital's medical records and face-to-face meetings.

The study enrolled adult outpatients diagnosed with CTS through clinical classification and nerve conduction study (NCS), who were deemed unsuitable for conservative (non-surgical) approaches. All patients who met the following inclusion criteria were considered: (i) Presented CTS symptoms (numbness, tingling, and pain); (ii) exhibited confirmed CTS signs during physical examination, including a positive Phalen or Tinel sign; and (iii) had CTS confirmed by NCS (prolonged distal motor latencies of the median nerve of >4.5 ms or sensory nerve conduction velocity of <40 m/s). However, this research excluded pregnant women, patients diagnosed with musculoskeletal disorders, peripheral neuropathy, or recurrent CTS, and patients who refused voluntary participation and/or had lost in follow-up with the Department of Orthopedics and Traumatology during the study period.

Nerve conduction studies were performed on both hands by trained clinical technicians, with patients positioned supine and in a relaxed state. Electrophysiological testing through NCS, as well as clinical presentations during pre-operative and post-operative follow-up periods, were used as the outcome parameters to assess the effectiveness of open CTR. The classification for the effectiveness assessment is as follows: If symptoms of CTS persist after surgery, the condition is categorized as persistent; should symptoms of CTS reappear within 3 months or more following the surgery, it is classified as recurrent; in cases where the outcomes show a transition from a severe to a mild or moderate stage of the disease, the treatment is considered successful[18]. Face-to-face follow-up visits were carried out depending on the patient's needs and the assessment of OCTR outcomes. OCTR procedures were performed at Zliten Teaching Hospital in accordance with the severity of the median nerve neuropathy and the recommended clinical guidelines. All surgical operations, patient assessments, history taking, and consultations were carried out by specialized orthopedic surgeons affiliated with Zliten Teaching Hospital.

The OCTR technique involved the administration of infiltrated local anesthesia during the surgical procedure. The open technique involved creating an approximately 3-cm incision aligned with the radial border of the ring finger, directly over the transverse carpal ligament in the proximal palm. Dissection proceeded through the palmar fascia until reaching the transverse carpal ligament, which was divided while being directly observed. In addition, the antebrachial fascia was released several centimeters proximal to the incision, and distally until yellow fat is observed. As part of the postoperative procedure, dressings were removed within 3 – 5 days, and hand range of motion exercises was promptly initiated. The sutures was removed after 10 days. Subsequently, the median nerve gliding exercise was started.

Using Krejcie and Morgan's table for sample size calculation, it was determined that a sample size of 260 was required for a population of 800 outpatients at the Department of Orthopedics and Traumatology. Ultimately, a total of 256 patients met the eligibility criteria and regularly attended follow-up sessions. Consequently, these 256 patients were included in the data analysis.

2.2. Statistical analysis

Data were entered and analyzed using the Statistical Package for the Social Sciences, version 23.0. Continuous variables were expressed as mean and standard deviation (SD) for normally distributed data. However, for non-normally distributed data, median and interquartile ranges were used. An independent *t*-test was performed when analyzing normally distributed data to assess differences in BMI between patients with and without post-operative complications. In addition, non-parametric tests, such as the Chi-square test, were conducted to evaluate the association between NCS severity and post-operative

complications. In addition, McNemar's test was used to perform pair-wise comparisons of outcome measurements between the pre-operative and post-operative phases. The statistical significance level was set at *P*-value less than 5% ($P < 0.05$).

2.3. Ethical consideration

The study adhered to ethical protocols in accordance with the Helsinki's Declaration, 2013 revision. All patients were duly informed about the study and were assured of the confidentiality of their data. Throughout the course of this study, every precaution was taken to prevent any harmor potential risks to the patients. Permission from patients was obtained through both verbal communication and the acquisition of written informed consent. No experimental intervention took place during or after the study. Furthermore, the study received full approval from the Scientific Research and Ethics Committee at Zliten Teaching Hospital in Libya.

3. Results

A total of 256 patients were included in this data analysis. Demographic information of study participants and clinical presentation is shown in Table 1. Descriptive statistics reveal that 127 patients (49.6%) presented bilateral CTS, 92 patients (35.9%) with right CTS, and 37 patients (14.5%) with left CTS. The age of the study participants ranged from 22 to 90 years, with a mean age of CTS presentation at 47 years (SD = 12.1). Of the participants, 204 (79.7%) were female, while 52 (20.3%) were male. The mean BMI was 28.08 m^2/kg (SD = 2.5), falling within the "overweight" range according to the World Health Organization classification.

The NCS results were assessed and classified according to the Bland classification, which is based on the electrophysiological severity of the condition. Following the NCS assessment, it was observed that 50.8% of the patients demonstrated severe compression of the median nerve. During the pre-operative stage, clinical examinations revealed that 95.0% of the CTS patients reported experiencing dull aching discomfort in their hand and forearm, accompanied by hand paresthesia and numbness. In addition, clumsiness was observed in 66 patients (25.8%), and thenar muscle atrophy was noted in 13 patients (5.0%).

Out of 256 patients, 232 (90.6%) were highly satisfied with the procedure and reported no recurrence of symptoms during the follow-up period. Conversely, 24 patients (9.4%) experienced post-operative complications. Three main complications were documented after OCTR during the follow-up period. Five patients (2.0%) encountered wound dehiscence (superficial infection), which was treated with dressings and antibiotics. Fifteen patients (5.9%) presented with recurrent CTS symptoms (three of them

Table 1. Demographic information and clinical presentation of patients

Variable/Clinical parameter	Mean (SD)	Frequency (n=256)	Percentage
Age	47 years (12.1)		
Gender			
Male		5220.3	
Female		204	79.7
BMI	28.08 m^2/kg (2.5)		
CTS presentation			
Bilateral CTS		12749.6	
Right CTS		9235.9	
Left CTS		37	14.5
Nerve conduction study assessment			
Mild median nerve compression		41	16.0
Moderate median nerve compression		85	33.2
Severe median nerve compression		130	50.8
CTS presentation at preoperative stage			
Dull aching discomfort in the hand and forearm with hand paresthesia and numbness		24395.0	
Clumsiness		6625.8	
Thenar muscle atrophy		13	5.0

Abbreviation: CTS: Carpal tunnel syndrome.

were treated with revision carpal tunnel release, and the rest were provided symptomatic relief). Finally, 4 patients (1.5%) reported dissatisfaction with the operation due to the persistence of symptoms without notable improvement in their hands. A summary of these findings is presented in Table 2.

The post-operative follow-up revealed a statistically significant reduction in dull aching discomfort in the hand and forearm, as well as paresthesia and numbness when compared to the pre-operative period (10.2% vs. 90.5%; $P < 0.0001$). However, no significant differences were observed during the follow-up compared to the pre-operative assessment in terms of the clumsiness associated with CTS (18.8% vs. 25.8%; $P = 0.256$) and the presence of thenar muscle atrophy (1.6% vs. 5.0%; $P = 0.194$), as shown in Table 3).

The result of this study showed no significant relationship ($P = 0.194$) between the presence of post-operative complications and BMI (Table 4). However, a statistically significant ($P = 0.011$) relationship was observed between the severity of NCS and the occurrence of post-operative complications (Table 5).

4. Discussion

Carpal tunnel release is a well-established treatment modality for patients with CTS. A variety of techniques, including endoscopic and OCTR, have been advocated for this purpose. OCTR is typically considered when minimally invasive measures prove insufficient in achieving effective CTS management through median nerved compression. To the best of our knowledge, this study represents the first prospective investigation conducted in Libya to explore the

Table 2. Postoperative outcomes and complications of OCTR surgery

Clinical presentation during post-operative follow-up	Frequency (n=256)	Percent (%)
No recurrence or complication	232	90.6
Recurrent CTS	15	5.9
Wound infections	5	2.0
Persistent symptoms with no improvement	4	1.5

Abbreviations: CTS: Carpal tunnel syndrome; OCTR: Open carpal tunnel release.

Table 3. Comparison of CTS symptoms at the pre-operative stage versus post-operative follow-up

Symptoms	Pre-operative stage (n [%])		Post-operative follow-up (n [%])		χ^2 statistic (df)	*P*-value[a]
	Yes	No	Yes	No		
Dull aching discomfort in the hand and forearm with hand paraesthesia and numbness	243 (95.0)	13 (5.1)	26 (10.2)	230 (89.8)	9.04 (1)	0.000*
Clumsiness	66 (25.8)	190 (74.2)	48 (18.8)	208 (81.3)	6.01 (1)	0.256
Thenar muscle atrophy	13 (5.0)	243 (95.0)	4 (1.6)	252 (98.4)	5.82 (1)	0.194

Notes: [a]McNemar's test; *$P<0.05$. CTS: Carpal tunnel syndrome.

Table 4. Differences in BMI between patients with and without post-operative complications

Post-operative complications	n (%)	Mean BMI (SD)	*t*-statistic	*P*-value[a]
Yes	24 (9.4)	28.6 (13.8)	1.98 (165)	0.143
No	232 (90.6)	25.8 (12.5)		

Note: [a]Independent *t*-test. BMI: body mass index; SD: Standard deviation.

Table 5. Association between NCS severity and post-operative complications

Post-operative complications	n (%)	NCS severity		χ^2 statistic (df)	*P*-value[a]
		Severe (n [%])	Mild–moderate (n [%])		
Yes	24 (9.4)	19 (79.2)	5 (20.8)	5.93 (1)	0.011*
No	232 (90.6)	111 (47.8)	121 (52.2)		

Notes: [a]Chi-square test; *$P<0.05$. NCS: Nerve conduction studies.

surgical outcomes of OCTR among CTS patients seeking treatment at Zliten Teaching Hospital.

In general, the implementation of OCTR procedures in CTS patients yielded favorable results during the post-operative follow-up. Surgical and post-surgical photographs of the hand of a female patient are presented for reference in Figures 1 and 2, respectively.

Approximately 9.4% of our study participants experienced postoperative complications, while 90.6% were highly satisfied with the results of the open release, reporting no recurrence symptoms. The review by Kuschner *et al.* noted an overall complication rate ranging from 1% to 2%[19]. In a study by Kulick *et al.*, 4.6% of 130 hands treated with the open release technique developed recurrent symptoms during post-operative follow-up[20].

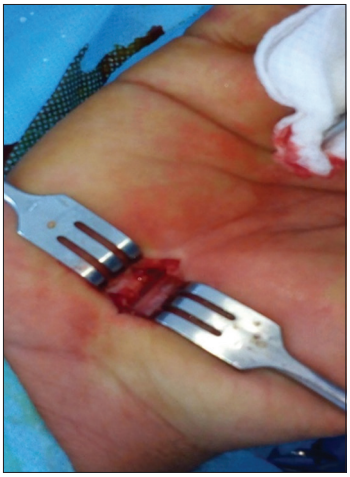

Figure 1. A photograph of the hand of a 53-year-old female patient with hyperemic median nerve during an open carpal tunnel release surgical procedure.

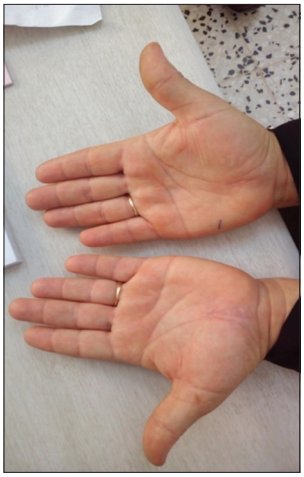

Figure 2. A photograph of the hand of the patient post-surgery (1-year follow-up).

Our study observed that 5.9% of patients presented with recurrent symptoms, and 1.5% experienced persistent symptoms even after the operation. A large-scale study involving 2053 open carpal tunnel decompressions revealed that only 34 patients (1.6% of cases) required re-exploration due to symptom recurrence[21]. However, it is worth noting that contrasting findings have been reported in other studies, with recurrent symptoms of CTS in approximately 19% of patients following OCTR, with up to 12% requiring re-exploration[18,22-24]. A study conducted in Iran reported an incidence of 12.4% for recurrent CTS and 10.4% for persistent CTS after OCTR[18]. Furthermore, other studies have reported that these symptoms can persist or recur in up to 30% of cases following surgery, and such cases are more likely to develop post-operative complications[24,25]. Therefore, it is evident that several patients prefer conservative treatments over surgical procedures due to their minimally invasive nature[26]. A previous study has documented several post-surgical adverse effects, including infections, painful or hypertrophic scars, wound hematomas, edema, wrist discomfort, stiffness, and reflex sympathetic dystrophy, affecting approximately 56.6% of CTS patients[27]. In comparison, our study identified only 2% of patients presenting with superficial wound infections, indicative of effective post-operative management by the hospital staff while treating the patients. The correlation between post-operative complications, symptom persistence, and partial median nerve decompression has been noted[18]. These findings support the effectiveness of our surgical strategy, contributing to the low complication and recurrence rates observed in our study. It is important to recognize that an increased incidence of complications and recurrence of symptoms occur after surgical failures, primarily attributed to diagnostic errors in the cases involving double crush in nerve entrapment syndromes, median nerve tumors, and iatrogenic injuries[28].

The discrepancy observed in the outcomes of various studies can be attributed to the demographic characteristics of the study participants. The previous literature has indicated that CTS is more prevalent among specific demographic groups, including women, obese individuals[15] and middle-aged individuals[14], those with metabolic and degenerative conditions[5], and certain occupational groups with repetitive wrist movements[29]. For instance, a review article discussed a study involving 60 patients treated with the open release technique, reporting that only one patient required CTS revision surgery during a mean follow-up period of 5.5 years[30]. In contrast, our study reported three revision operations within a 2-year follow-up period. The primary factors contributing to CTS recurrence included inadequate surgical skill, incorrect

diagnosis[18], and incomplete relaxation of the distal portion of the transverse carpal ligament[29]. Therefore, removing adequate TCL is a crucial first step in promoting the success of CTS surgery, resulting in complete median nerve decompression, symptom alleviation, and a reduced recurrence rate. According to the results of a multivariable logistic regression model, other factors that contributed to a higher revision rate included age, male sex, bilateral carpal tunnel release, and ECTR. In addition, it was shown that pre-operative splint therapy reduced the likelihood of requiring revision surgery, while smoking and rheumatoid arthritis increased the cumulative risk[31].

During the pre-operative stage, it was observed that 50.8% of the patients exhibited severe nerve compression. Subsequently, the post-operative follow-up revealed a significant reduction in the dull aching discomfort in the hand and forearm, as well as paresthesia and numbness. Although there were no significant changes in terms of clumsiness and thenar atrophy, notable symptomatic relief was evident after the surgery. A recent study conducted in Pakistan[32] also reported similar findings, where the majority of participants (55.8%) presented with severe CTS during pre-operative assessments. However, post-operative results indicated that 96%of them achieved symptomatic relief, with only 3.9% reporting persistent symptoms. It is worth noting that post-operative complications were found to be significantly linked to CTS severity, but not to the patients' BMI. While earlier research[14,15] established a substantial correlation between higher BMI and the occurrence of CTS, the underlying pathophysiological mechanisms remain not fully understood[15]. Furthermore, the high BMI levels were not found to be associated with CTS severity[15], which is a rather surprising finding. In contrast, another study[5] reported that surgical outcomes for diabetic patients may vary due to the direct influence of the disease itself on the clinical severity of CTS. The post-operative reduction in the CTS Assessment Questionnaire (CTSAQ) score (<1.04) among patients signifies a clinically significant improvement in their health[33]. Therefore, it can be inferred that a comprehensive diagnosis and severity assessment plausibly play an important role in achieving the optimal treatment modality for CTS patients.

From a pathophysiological perspective, increased pressure within the carpal tunnel is a major contributing factor to the etiology of CTS. The heightened pressure impairs blood supply to the median nerve, eventually resulting in nerve damage and the clinical presentation of symptoms. A significant proportion of CTS patients suffer from these symptoms due to repetitive motion injuries of the wrist[29]. The presence of ischemia and fibrotic nerve fiber bundles may contribute to persistent neurological symptoms, which are related to partial permanent damage to the median nerve. The persistence of preoperative CTS symptoms frequently hinders the achievement of successful surgical outcomes[30]. Unsatisfactory surgical results are typically attributed to the incomplete sectioning of the TCL distal tract or, less commonly, the distal portion of the antebrachial fascia[29]. It is imperative to ensure complete incision of the TCL, regardless of the surgical procedure chosen, to achieve median nerve relief in carpal tunnel surgery, and reduce the rates of recurrence[29].

4.1. Limitations and recommendations

While efforts were made to minimize confounding effects related to differences in demographic factors such as occupation and comorbid status, it is important to acknowledge that a majority of the research participants were female in their 40s, which may have influenced the study's findings. Therefore, future research should aim to address this limitation by working with more homogenous patient groups to mitigate the impact of confounders. Our study focused on assessing post-operative patient-reported outcomes using the CTSAQ tool, specifically measuring symptoms, severity, and function related to CTS. However, it failed to identify any long-term disabilities among patients who experienced post-operative complications or symptom recurrence. Our study employed a case series approach with the primary objective of investigating the surgical outcomes of OCTR, and as such, we did not conduct subgroup analyses to compare at-risk groups. However, it would be valuable for future research to explore the correlation between post-operative complications and demographic variables such as age, gender, occupation, and comorbid status to uncover significant findings. Therefore, future researchers should focus on addressing the association between post-operative complications and demographic factors. Surgeons are encouraged to prioritize a minimally invasive open release approach over the early reduction of immobility and pain during the initial post-operative days. This approach promotes better visualization of neurovascular structures and increases the likelihood of achieving a complete release, resulting in long-term symptomatic relief and a reduced risk of recurrence[34]. Furthermore, future studies are recommended to compare the clinical outcomes of new surgical interventions or approaches in CTS treatment. Likewise, it is important to consider the role of conservative rehabilitative measures, such as physical therapy, during post-operative follow-up. This can effectively manage CTS symptom recurrence, facilitate faster recovery, and restore maximum wrist function.

5. Conclusion

The treatment of CTS is always aimed at attaining a significant reduction in CTS symptoms postoperatively. While the etiology of CTS may be multifactorial and attributable to different factors, our study did not reveal any statistically significant relationship between BMI and postoperative complications. However, a significant association was observed between the severity of NCS and post-operative complications. Our results indicated positive clinical outcomes, underscoring the strong recommendation for the use of OCTR methods in the treatment of CTS.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Mohamed Gwila,Mostafa Ebshina
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Investigation: Mohamed Gwila,Mostafa Ebshina, Syeda Humayra
Methodology: Mohamed Gwila
Writing – original draft: Mohamed Gwila
Writing – review & editing: Syeda Humayra, Moniruddin Chowdhury

Ethics approval and consent to participate

The study followed the ethical protocols in accordance with the Helsinki's Declaration, 2013 revision. The study has been approved by the Scientific Research and Ethics Committee at Zliten Hospital in Libya (Approval ID: ZHEC-2015/1209-038).

Consent for publication

All patients were informed about the study, and their data confidentiality was ensured. The patients were not exposed to any harmful events or potential risks throughout this study. Permission was obtained from patients both verbally and in the form of written informed consent. No experimental intervention took place during or after the study.

Availability of data

Data can be obtained from corresponding author following formal request.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Prognostic evaluation of relapse based on squamous cell carcinoma antigen, CXCR2, and CD44V6 blood levels in patients with Stage I–II squamous cell lung cancer

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Abstract

Lung cancer, the leading cause of cancer-related mortality, predominantly exists as non-small cell lung cancer, accounting for approximately 85% of cases and comprising adenocarcinoma, squamous cell carcinoma (SCC), and large cell carcinoma as the three most prevalent histological subtypes. This study focused on investigating pre- and post-operative concentrations of SCC antigen in blood serum, as well as the percentage of CXCR2-containing lymphocytes and CD44v6-containing monocytes in blood cell populations among patients with Stages I–II squamous cell lung cancer (SCLC) within 1 year after tumor resection. The primary objective was to assess their potential for predicting relapse. The study cohort comprised 57 patients (32 men and 25 women) with newly diagnosed squamous cell lung cancer (21 at stage I and 36 at stage II). Following tumor resection, categorized as R0 in terms of surgical intervention, all parameters were examined before surgery and at 3 weeks, 3 months, and 6 months postoperatively. Analysis revealed that the probability of relapse could be accurately predicted, ranging from 68.4% to 89.5%, based on differences in SCC antigen concentration, the percentage of lymphocytes with CXCR2, and monocytes with the CD44v6 receptor during various post-operative intervals. Subsequent regression analysis and the formulation of a combined model incorporating the above-mentioned parameters led to an enhanced predictive value for tumor recurrence, reaching 96.5% accuracy (with specificity at 95.6% and sensitivity at 100%). These results indicate the potential utility of the combined model as an additional marker for predicting postoperative relapse in patients with Stage I–II SCLC.

Keywords: Squamous cell lung cancer; Squamous cell carcinoma; CXCR2; CD44v6; Post-operative relapse; Prognosis

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1. Introduction

Lung cancer stands as the primary contributor to cancer-related mortality in men, with women ranking it as the second leading cause[1]. Non-small cell lung cancer (NSCLC)

constitutes the predominant form of lung cancer, accounting for approximately 85% of cases. The three most prevalent histological subtypes of NSCLC include adenocarci 70% of patients with Stage I NSCLC and 35 – 40% with Stage II (according to TNM classification) noma (AC), squamous cell carcinoma (SCC), and large cell carcinoma[2]. In the early stages, only 60– overcome the 5-year survival barrier. The basis of treatment for such patients (Stages I–IIIA) is tumor resection during surgery. Patients usually undergo surgery in the amount of R0, which involves the complete removal of the tumor tissue and metastases. However, during post-operative observation, relapse is diagnosed in 20% of patients at Stage I and in 51% at Stage II, mainly due to metastases development after resection[3,4]. Performing an R0 operation, the surgeon always assumes that the tumor and existing metastases were completely removed. However, there is no guarantee that tumor cells, even in a single quantity, or micrometastases could remain in the surrounding tissue. This lack of guarantee is the reason for the development of tumor relapses after resection. The monitoring algorithm for patients with NSCLC includes a physical examination every 3 months for the first 2 years after surgery, and computed tomography (CT) scan is performed at 6 and 12 months after treatment[5]. Essentially, this is the timing of early relapse detection. Meanwhile, a relapse can occur up to 6 months or during the period 6 months to 1 year after surgery[4]. Thus, there is a need to search for predictors that can serve as an important addition to ongoing monitoring to identify or predict tumor recurrence in each individual patient after surgical treatment as early as possible.

The pre-operative levels of tumor proteins in serum and blood cells have proven to be predictive of high or low relapse-free survival for patients with AC and SCC separately[6]. Despite both belonging to the NSCLC group, these histological subtypes exhibit significant differences[1,7]. AC typically manifests with peripheral localization of the tumor process, while SCC tends to develop in the central regions of the lung[1]. More than 70% of AC cases are associated with mutations in the epidermal growth factor receptor (*EGFR*) gene and anaplastic lymphoma kinase (CD246), while no identified gene polymorphisms are associated with the development of SCC[7]. In contrast to AC, SCC predominantly develops in smokers[1]. Therefore, the separation of NSCLC patients based on their histological types appears to be more appropriate. Building on our previous investigations, the most reliable prognostic indicators for SCC include SCC antigen, the percentage of lymphocytes with the CXCR2 receptor (CXCR2 [% in lymphocytes]) in the total population of these blood cells, and the percentage of monocytes with CD44v6 receptor (CD44v6 [% in monocytes])[6].

It is well-established that the SCC antigen functions as an inhibitor of certain intracellular serine proteinases, thereby conferring resistance to apoptotic factors in tumor cells[8]. The CXCR2 receptor serves as a receptor for the chemokine CXCL5, and its intense expression by leukocyte cells plays a role in inflammatory processes associated with tumor growth[9]. In addition, the CD44v6 receptor is implicated in cell adhesion and contributes to the dissemination of tumor cells[10]. The previous studies have indicated that the combined assessment of these parameters yields the most prognostic information compared to their individual evaluation[6]. This article aims to present an evaluation of these markers in a dynamic context, both before and after tumor resection, with the aim of identifying predictive indicators for assessing the likelihood of relapse during the first year after treatment in patients with Stages I–II NSCLC.

2. Materials and methods

2.1. Study population

The characteristics of the patients are presented in Table 1.

2.2. Ethical approval and consent

All patients provided written voluntary consent to participate in the study. The study received approval from the Biomedical Ethics Committee of Belarusian State Medical University (Committee meeting No. 2 dated 10/04/2021).

2.3. Study design and sample collection

The study involved 57 patients (32 men and 25 women) admitted to the clinic of the N.N. Alexandrov National Cancer Center of Belarus between 2021 and 2022, all of whom received an initial diagnosis of Stage I or II NSCLC. All patients underwent surgical resection of the tumor (surgical volume - R0). Post-treatment monitoring for each patient included a physical examination every 3 months throughout the 1st year post-surgery. In the absence of patient complaints and signs of disease during the physical examination, CT scans were performed at 6 and 12 months after treatment, constituting the essential timeframe for early relapse detection. Information concerning relapse development in the examined patients after surgery was obtained from CT data in the Cancer Register of the Republic of Belarus (via the N.N. Alexandrov National Cancer Center of Belarus). Candidate biomarkers were measured in all patients before treatment and at 3 weeks, 3 months, and 6 months post-surgery. Blood obtained on an empty stomach from the cubital vein of patients was collected into a vacutainer with EDTA-K2 as an anticoagulant for this purpose.

Table 1. Characteristics of patients included in the study

Characteristics	Frequency (n [%])
Number of patients (N)	57
Age (M±σ years)	58±20.5
<40	2 (3.5)
41–50	9 (15.8)
51–60	27 (47.4)
61–70	14 (24.6)
>70	5 (8.7)
Gender (male/female)	32/25
Smoking status (male, former/current/never)	7/23/2
Smoking status (female, former/current/never)	5/17/3
Tumor characteristics	
Stage (based on the eighth edition of TNM staging of lung cancer)	
I	21 (36.8)
II	36 (63.2)
Prevalence of the tumor process	
T1	15 (26.4)
T2a	21 (36.8)
T2b	13 (22.8)
T3	8 (14.0)
Damage to regional lymph nodes	
N0	37 (64.9)
N1	20 (35.1)
Degree of tumor differentiation	
G I	11 (19.3)
G II	29 (50.9)
G III	17 (29.8)
Localization	
Right lung	33 (57.9)
Left lung	24 (42.1)
Median recurrence-free survival (months)	25.3

Serum was obtained by placing blood into a tube with thrombin and separating gel.

2.4. Analysis of samples

The concentration of SCC antigen in blood serum was determined using the electrochemiluminescent method on a Cobas e411 automated analyzer (Roche Diagnostics GmbH, Germany) using original Elecsys SCC kits (Roche Diagnostics GmbH, Germany). To assess the concentration of CXCR2 and CD44v6 receptors in leukocyte cells and the density of their location in the cell membrane, a Navios flow cytometer (Beckman Coulter, USA) was employed. For this analysis, 100 µl of blood and a solution containing a mixture of antibodies with fluorescent labels were combined in a test tube. The antibodies included CD44v6-FITC (Sigma-Aldrich, USA), CD182(CXCR2)-PE (BioLegend, USA), and CD45-Pacific Orange (Exbio, Czech Republic). Following a 15-min incubation in the dark with these fluorescent-labeled antibodies, 1 ml of VersaLyse lysis solution (Beckman Coulter, France) was added to the mixture. Antibodies were then fixed on the cell surface using the IQTest3 fixing solution (Beckman Coulter, France).

2.5. Statistical analysis

To determine the relationship between the duration of the relapse-free period and observation time, Kaplan–Meier plots were constructed. Group comparisons based on different relapse-free survival durations were performed using the log-rank test and Chi-square (χ^2). The relationship between changes in the level of measured indicators and survival was assessed through uni- and multivariate Cox proportional hazards models.

The integral information content of laboratory tests was assessed using the method of constructing characteristic receiver operating characteristic (ROC) curves, followed by the calculation of the area under the ROC curve (AUC). Prognostic values of the analyzed indicators were determined by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and test accuracy. These calculations involved the true positive, true negative, false positive, and false negative results of the test, following generally accepted formulas[11]. The threshold value was determined as the optimal combination of sensitivity and specificity when constructing sensitivity curves versus the probability of false-positive results.

The combined model for predicting the probability of NSCLC relapse integrated the results of calculating the regression equation (Y), which included the concentration of SCC antigen in blood serum (X1), percentages of lymphocytes with CXCR2 (X2), and monocytes with CD44v6 (X3) in the populations of these blood cells[6]:

$$Y = \frac{\exp(-0,492 + 0,306 \times X1 + 0,759 \times X2 + 0,917 \times X3)}{1 + \exp(-0,492 + 0,306 \times X1 + 0,759 \times X2 + 0,917 \times X3)} \quad (I)$$

For all types of statistical analyses, the critical value of the significance level was set at 5%.

3. Results

In the majority of patients (42 out of 57), 3 weeks after surgical tumor removal, the level of SCC antigen, the

percentage of lymphocytes with the CXCR2 receptor, and the percentage of monocytes with the CD44v6 receptor in the total population of these blood cells, as well as the results of the calculated combined model, decreased to values below the threshold values (TV) (TV: SCC = 1.51 ng/ml; CXCR2 [% in lymphocytes] = 10.7%; CD44v6 [% in monocytes] = 2.20%; combined model=0.213[6]). The most pronounced decrease was observed for the combined model (39.1%), while for individual indicators, their TVs did not exceed 32.0%. In this group, relapse developed in only three cases during the 1-year observation period.

In the remaining 15 of the 57 patients examined, the levels of the studied indicators 3 weeks after surgical treatment also decreased but remained above TVs. For these patients, the initial median drop at 3-week postoperatively for the individual outcome ranged from 36.9% to 45.1%, and for the combined model outcome, it was 48.9%. Tumor recurrence occurred within a year in nine out of these 15 patients, as diagnosed based on CT results (Table 2).

The first relapses in the group of patients with an elevated post-operative SCC antigen level were diagnosed 3.9 months after surgery, whereas in those with a normal post-operative level, relapses occurred later, after 8.5 months (Figure 1A). Examining the level of CXCR2 (% in lymphocytes) 3 weeks after resection as a prognostic criterion revealed that exceeding the TV at this time was also diagnosed in a patient who developed a relapse after 3.9 months (Figure 1B). However, in the patient experiencing a relapse 8.3 months after surgery, the level of the indicator 3 weeks postoperatively was below the TV, classifying them into a low-risk group for relapse. Stratifying patients into high- and low-risk groups based on CD44v6 (% in monocytes) 3 weeks after surgery showed a patient from the low-risk group developing a relapse after 6.3 months. In contrast, a patient with a relapse after 3.9 months exhibited an increased level of the indicator, along with SCC antigen and CXCR2 (% in lymphocytes) (Figure 1C). The combined model, incorporating all three aforementioned indicators, demonstrated superior predictive ability. For a patient with a relapse diagnosed after 3.9 months, the calculation result exceeded TV 3 weeks after surgery. The first relapse in patients with a value below the threshold 3 weeks after

Table 2. Changes in the concentration of SCC antigen, the percentage of lymphocytes with the CXCR2 receptor, and monocytes with the CD44v6 receptor in the populations of these blood cells in patients who developed relapse (12 out of all 57 patients)

Patient	Index	Before treatment	After tumor resection			Time to relapse (months)
			3 weeks	3 months	6 months	
1	SCC antigen (ng/ml)	2.47	1.55	1.73	2.12	3.9
	CXCR2 (% in lymphocytes)	19.30	10.80	13.40	17.65	
	CD44v6 (% in monocytes)	4.20	2.56	2.93	3.51	
	Combined model	0.503	0.257	0.341	0.467	
2	SCC antigen (ng/ml)	2.71	1.73	1.90	2.21	4.3
	CXCR2 (% in lymphocytes)	17.15	10.25	13.00	17.10	
	CD44v6 (% in monocytes)	2.15	2.20	2.55	3.10	
	Combined model	0.434	0.231	0.313	0.421	
3	SCC antigen (ng/ml)	3.75	2.89	3.05	3.36	5.6
	CXCR2 (% in lymphocytes)	20.90	10.90	13.65	17.80	
	CD44v6 (% in monocytes)	4.85	3.85	4.20	4.75	
	Combined model	0.598	0.273	0.355	0.466	
4	SCC antigen (ng/ml)	1.93	1.56	1.73	2.04	6.3
	CXCR2 (% in lymphocytes)	17.4	13.30	16.15	20.40	
	CD44v6 (% in monocytes)	3.85	2.10	2.55	3.30	
	Combined model	0.429	0.22	0.307	0.422	
5	SCC antigen (ng/ml)	2.02	1.61	1.79	2.12	7.2
	CXCR2 (% in lymphocytes)	18.1	14.2	17.1	21.3	
	CD44v6 (% in monocytes)	3.90	2.40	2.80	3.45	
	Combined model	0.501	0.227	0.313	0.434	

(Cont'd...)

Table 2. ***(Continued)***

Patient	Index	Before treatment	After tumor resection			Time to relapse (months)
			3 weeks	3 months	6 months	
6	SCC antigen (ng/ml)	2.15	1.63	1.82	2.23	7.6
	CXCR2 (% in lymphocytes)	19.70	14.30	17.25	21.75	
	CD44v6 (% in monocytes)	3.75	2.05	2.55	3.15	
	Combined model	0.493	0.231	0.322	0.446	
7	SCC antigen (ng/ml)	2.07	1.61	1.76	2.06	8.3
	CXCR2 (% in lymphocytes)	16.60	10.50	13.30	17.30	
	CD44v6 (% in monocytes)	4.05	2.35	2.80	3.45	
	Combined model	0.477	0.248	0.337	0.468	
8	SCC antigen (ng/ml)	1.92	1.48	1.64	2.07	8.5
	CXCR2 (% in lymphocytes)	16.50	10.60	13.60	18.50	
	CD44v6 (% in monocytes)	4.15	2.95	3.50	4.10	
	Combined model	0.491	0.229	0.322	0.457	
9	SCC antigen (ng/ml)	2.13	1.52	1.68	2.17	9.5
	CXCR2 (% in lymphocytes)	17.10	10.75	13.70	18.40	
	CD44v6 (% in monocytes)	4.10	2.25	2.65	3.40	
	Combined model	0.407	0.212	0.31	0.449	
10	SCC antigen (ng/ml)	2.27	1.73	1.88	2.21	9.8
	CXCR2 (% in lymphocytes)	18.20	11.55	14.40	18.65	
	CD44v6 (% in monocytes)	4.25	2.85	3.20	3.85	
	Combined model	0.483	0.25	0.344	0.485	
11	SCC antigen (ng/ml)	1.80	1.37	1.57	1.89	10.3
	CXCR2 (% in lymphocytes)	19.10	10.85	14.1	18.6	
	CD44v6 (% in monocytes)	3.85	2.10	2.50	3.20	
	Combined model	0.397	0.206	0.305	0.433	
12	SCC antigen (ng/ml)	1.85	1.33	1.49	1.91	11.5
	CXCR2 (% in lymphocytes)	15.90	10.60	13.45	18.35	
	CD44v6 (% in monocytes)	3.75	2.15	2.55	3.25	
	Combined model	0.405	0.209	0.295	0.433	

Abbreviation: SCC: Squamous cell carcinoma.

surgery occurred after 9.5 months (Figure 1D). The larger value of the χ^2 criterion, determined from the results of the log-rank test, for the combined model (Figure 1D) further confirms its superior predictive ability compared to using individual indicators for patient stratification (Figure 1A-C).

The application of a univariate Cox model revealed that, for all measured parameters, the hazard ratios exceed 1, and the confidence interval values do not cross the value of 1 (Table 3). This observation underscores the existence of an association between the individual parameter levels and the combined determination's values, 3-week post-surgery, with the risk of relapse.

The obtained data indicate that predicting tumor recurrence based on the levels of all measured parameters 3-week post-resection is feasible by adopting a TV criterion. However, the prognostic accuracy is constrained, as evidenced by relapse occurrence in three out of 12 cases.

Notably, in patients devoid of relapse during the 1-year observation period, the levels of all measured parameters, reduced to TV 3-week post-surgery, exhibited minimal changes at subsequent intervals—specifically, at 3 and 6 months (Figure 2). Conversely, in the patients experiencing relapse, a significant elevation in the levels of all analyzed indicators transpired during these periods. For instance, SCC antigen concentrations rose to 1.73 ng/ml

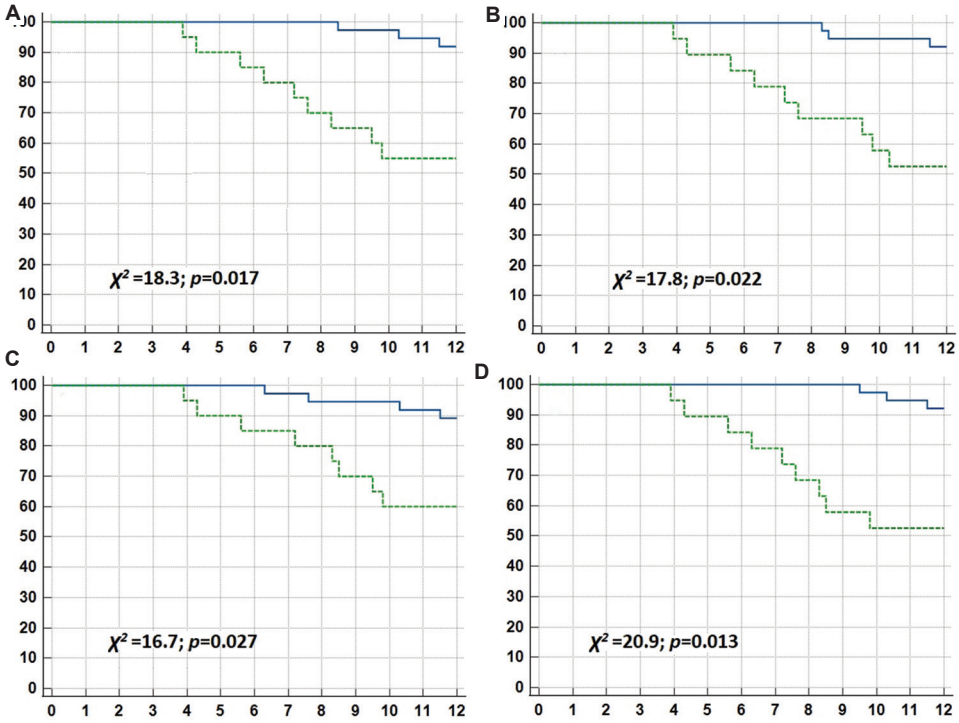

Figure 1. Relapse-free survival of patients depending on the level of the indicator 3 weeks after surgery: (A) Squamous cell carcinoma antigen, (B) CXCR2 (% in lymphocytes), (C) CD44v6 (% in monocytes), (D) combined model. Legends: X-axis: Time (months); Y-axis: Disease-free survival (%); Solid line on the graph: Low probability of relapse; dashed line: High probability of relapse.

at 3-month post-surgery, compared to 1.33 ng/ml 3 weeks after resection (Figure 2A). At 6-month post-surgery, the SCC antigen content in the blood of these patients increased further to 2.05 ng/ml. Similar upward trends in indicators were observed for CXCR2 (% in lymphocytes), CD44v6 (% in monocytes), and the combined model (Figure 2B–2D).

The changes in the level of the measured indicators are further elucidated by the disparity in their post-operative values during the specified periods (Table 4). In all parameters, the difference in values at 3 months post-tumor resection was significantly higher than that observed at 3-week post-surgery. Specifically, the increase for SCC antigen averaged 194% (Figure 2A), for CXCR2 (% in lymphocytes) – 149% (Figure 2B); for CD44v6 (% in monocytes) – 150% (Figure 2C), and the combined model – 144% (Figure 2D). Subsequently, at 6 months compared to 3-week post-surgery, the increments were even more pronounced, reaching 306%, 250%, 270%, and 240%, respectively.

Cox proportional hazards models delineate the relationship between the difference in the values of the measured parameters during the periods of 3 weeks–3 months, 3–6 months, and 3 weeks–6 months after treatment, with a focus on relapse. According to the obtained data, the hazards ratio coefficient consistently exceeds 1 in all cases, and the confidence interval does not cross the value of 1 (Table 5). Notably, for the combined model, the hazards ratio value, whether in the univariate or multivariate models, was the highest. This observation underscores that the combined model exhibits a more robust relationship with the development of tumor recurrence.

The results of the ROC analysis allow us to determine TVs for the dynamics of changes (Figure 3, Tables 6-8).

The accuracy of predicting the likelihood of relapse, based on determining the difference in the level of each indicator separately during the 3 weeks – 3 months post-surgery periods, ranges from 68.4% – 77.2% (Table 6). The calculation of the combined model within this specified timeframe enhances the accuracy to 84.2%. This observation signifies that if the result of the combined model, calculated based on the level difference in this timeframe, exceeds 0.085, then in 58.8% of cases (PPV), the patient has a significantly high probability of tumor recurrence, while an equation value of ≤0.085 (TV) in 95.0% of patients (NPV) will correctly predict the absence of relapse.

The difference in the results of determining each of the indicators during the 3 months – 6 months post-treatment

period demonstrates the accuracy of predicting relapse, ranging from 71.9% to 82.5% (Table 7). The accuracy of the calculation result for the combined model within this specified timeframe is 91.2%. At the same time, PPV is 73.3%, NPV is 97.6%, and TV is 0.119.

Table 3. Relationship between the levels of measured indicators 3 weeks after surgery and the relapse-free survival of patients with Stage I–II NSCLC (univariate Cox proportional hazards model)

Index	HR (95% CI)	*P*
SCC antigen (ng/ml)	1.063 (1.004 – 1.122)	0.038
CXCR2 (% in lymphocytes)	1.037 (1.005 – 1.069)	0.031
CD44v6 (% in monocytes)	1.019 (1.012 – 1.026)	0.029
Combined model	1.103 (1.027 – 1.179)	0.019

Abbreviations: CI: Confidence interval; HR: Hazards ratio; *P*: An indicator of the level of statistical significance of the hazards ratio, SCC: Squamous cell carcinoma.

A more substantial difference in the levels of indicators during the 3 weeks – 6 months post-surgical treatment, compared with the 3 – 6 months period, carries a higher prognostic information value. Within the 3 weeks – 6 months timeframe, changes in their levels enable the prediction of the development of relapse within a year after treatment with an accuracy ranging from 86.0% – 89.5%, and for the combined model, the accuracy reaches 96.5% (Table 8).

4. Discussion

The scope of tumor resection, denoted as R0, necessitates the complete removal of the tumor and any developed metastases. Despite concerted efforts to eliminate tumor cells entirely, some may persist in the body. The possibility of this occurrence is significant, given that even the most advanced research methods may fail to detect these residual cells. Therefore, certain patients experience tumor relapses,

Table 4. Difference in postoperative levels of measured parameters in the blood of patients with tumor recurrence

Index	Time interval (median [25;75 percentiles])		
	3 weeks–3 months	3 months–6 months	3 weeks–6 months
SCC (ng/ml)	0.17 (0.16; 0.18)	0.33 (0.31; 0.41)[1]	0.52 (0.48; 0.58)[1,2]
CXCR2 (% in lymphocytes)	2.85 (2.80; 2.95)	4.25 (4.19; 4.55)[1]	7.10 (7.01; 7.68)[1,2]
CD44v6 (% in monocytes)	0.40 (0.37; 0.51]	0.60 (0.55; 0.65)[1]	1.08 (0.98; 1.15)[1,2]
Combined model	0.088 (0.085; 0.093)	0.127 (0.119; 0.138)[1]	0.217 (0.205; 0.227)[1,2]

Notes: [1]Statistical significance of the difference of indicator levels for a period of 3 – 6 months compared to 3 weeks – 3 months; [2]Statistical significance of the differences of indicator levels for a period of 3 weeks – 6 months compared to 3 weeks – 3 months.

Table 5. Cox proportional hazards model of the dependence of relapse-free survival of patients with NSCLC on the difference in the level of indicators after surgical treatment

Index	Time interval	Univariate model		Multivariate model	
		HR (95% CI)	*P*	HR (95% CI)	*P*
SCC (ng/ml)	3 weeks – 3 months	1.188 (1.003–1.373)	0.031	1.114 (1.004–1.224)	0.033
	3 months – 6 months	1.245 (1.012–1.478)	0.022	1.221 (1.098–1.344)	0.024
	3 weeks – 6 months	1.425 (1.037 – 1.813)	0.011	1.367 (1.015 – 1.719)	0.013
CXCR2 (% in lymphocytes)	3 weeks – 3 months	1.025 (1.003 – 1.047)	0.033	1.057 (1.003 – 1.111)	0.034
	3 months – 6 months	1.051 (1.005 – 1.097)	0.027	1.071 (1.009 – 1.133)	0.028
	3 weeks – 6 months	1.085 (1.008 – 1.162)	0.019	1.099 (1.015 – 1.183)	0.021
CD44v6 (% in monocytes)	3 weeks – 3 months	1.158 (1.025 – 1.291)	0.027	1.111 (1.018 – 1.204)	0.031
	3 months – 6 months	1.221 (1.028 – 1.414)	0.025	1.245 (1.029 – 1.461)	0.026
	3 weeks – 6 months	1.302 (1.099 – 1.505)	0.020	1.298 (1.079 – 1.517)	0.023
Combined model	3 weeks – 3 months	1.421 (1.102 – 1.740)	0.010	1.315 (1.099 – 1.531)	0.014
	3 months – 6 months	1.688 (1.158 – 2.118)	0.008	1.586 (1.118 – 2.051)	0.011
	3 weeks – 6 months	1.786 (1.148 – 2.424)	0.007	1.642 (1.107 – 2.177)	0.009

Notes: SCC: Squamous cell carcinoma; HR: Hazards ratio; CI: Confidence interval; *P*: An indicator of the level of statistical significance of the hazards ratio.

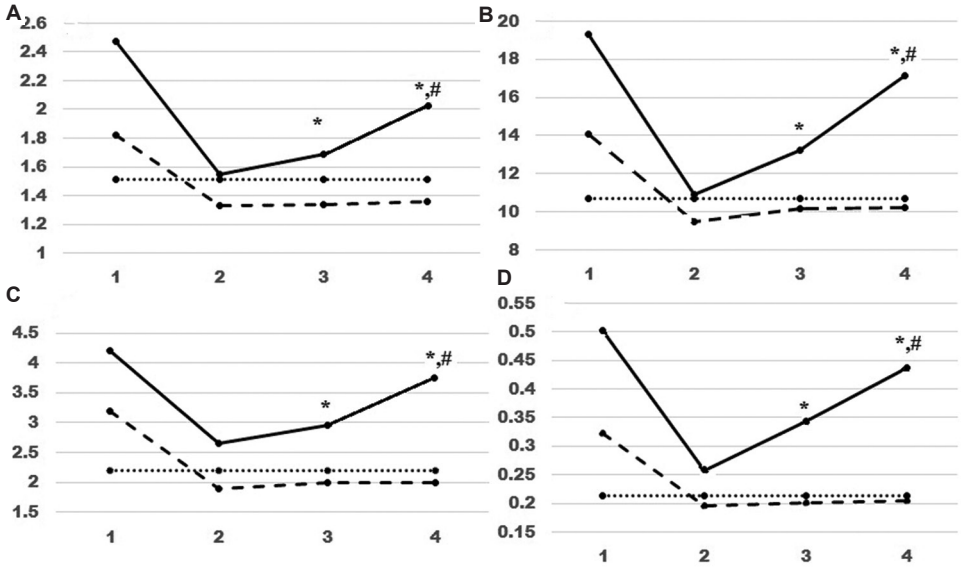

Figure 2. Dynamics of measured parameter values in patients with non-small cell lung cancer. (A) Squamous cell carcinoma antigen, (B) CXCR2 (% in lymphocytes), (C) CD44 (% in monocytes), and (D) combined model. Legends: Solid line: relapse cases; dashed line: non-relapse cases; dotted line: Threshold value. X-axis: 1: Before surgery; 2: 3 weeks after surgery; 3: 3 months after surgery; 4: 6 months after surgery. *Significant differences in the levels of indicators in the blood after 3 months compared to the level in the same patients 3 weeks after resection; #significant differences in the levels of indicators in the blood after 6 months compared to the levels in the same patients 3 weeks after surgery.

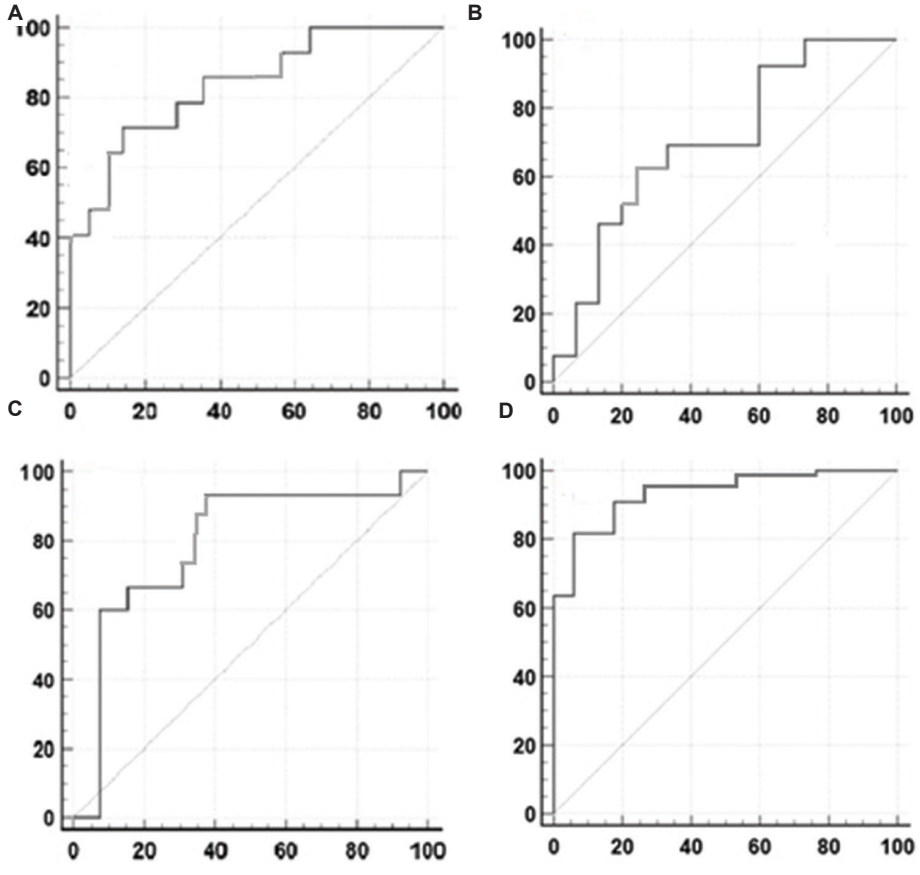

Figure 3. Receiver operating characteristic curves for differences in performance levels at 3-week and 3-month post-surgery. (A) Squamous cell carcinoma, (B) CXCR2 (% in lymphocytes), (C) CD44v6 (% in monocytes), (D) combined model. Legends: X-axis: Specificity; Y-axis: Sensitivity.

Table 6. Prognostic values of determining the difference in the levels of SCC antigen, CXCR2 (% in lymphocytes), CD44v6 (% in monocytes), and the combined model during the 3 weeks – 3 months post-treatment period for predicting the development of postoperative relapse in non-small cell lung cancer

Index	TV	SE	SP	PPV	NPV	AUC	ACC
SCC antigen (ng/ml)	0.16	75.0	77.8	47.4	92.1	0.702	77.2
CXCR2 (% in lymphocytes)	2.82	66.7	68.9	36.4	88.6	0.641	68.4
CD44v6 (% in monocytes)	0.38	58.3	73.3	36.8	86.8	0.683	70.2
Combined model	0.085	83.3	84.4	58.8	95.0	0.799	84.2

Notes: ACC: Accuracy; AUC: Area under ROC-curve; NPV: Predictive value of a negative result; PPV: Predictive value of a positive result; SCC: Squamous cell carcinoma; SE: Sensitivity; SP: Specificity; TV: Threshold value of the indicator based on the results of ROC analysis.

Table 7. Prognostic values of determining the difference in the levels of SCC antigen, CXCR 2 (% in lymphocytes), CD44v6 (% in monocytes), and the combined model during the 3 – 6 months post-treatment period for predicting the development of postoperative relapse in non-small cell lung cancer

Index	TV	SE	SP	PPV	NPV	AUC	ACC
SCC antigen (ng/ml)	0.32	83.3	82.2	55.6	94.9	0.793	82.5
CXCR2 (% in lymphocytes)	4.20	75.0	71.1	40.9	91.4	0.692	71.9
CD44v6 (% in monocytes)	0.58	66.7	75.6	42.1	89.5	0.708	73.7
Combined model	0.119	91.7	91.1	73.3	97.6	0.866	91.2

Notes: ACC: Accuracy; AUC: Area under ROC-curve; NPV: Predictive value of a negative result; PPV: Predictive value of a positive result; SCC: Squamous cell carcinoma; SE: Sensitivity; SP: Specificity; TV: Threshold value of the indicator based on the results of ROC analysis.

Table 8. Prognostic values of determining the difference in the levels of SCC antigen, CXCR2 (% in lymphocytes), CD44v6 (% in monocytes), and the combined model during the period 3 weeks – 6 months post-treatment period for predicting the development of postoperative relapse in non-small cell lung cancer

Index	TV	SE	SP	PPV	NPV	AUC	ACC
SCC antigen (ng/ml)	0.47	91.7	88.9	68.8	97.6	0.831	89.5
CXCR2 (% in lymphocytes)	7.05	83.3	86.7	62.5	95.1	0.815	86.0
CD44v6 (% in monocytes)	1.01	75.0	84.4	56.3	92.7	0.794	82.5
Combined model	0.195	100	95.6	85.7	100.0	0.928	96.5

Notes: ACC: Accuracy; AUC: Area under ROC-curve; NPV: Predictive value of a negative result; PPV: Predictive value of a positive result; SCC: Squamous cell carcinoma; SE: Sensitivity; SP: Specificity; TV: Threshold value of the indicator based on the results of ROC analysis.

primarily within the 1st year post-surgery[1]. Ongoing research endeavors are directed toward identifying effective criteria for detecting residual tumor cells in the body.

Our analysis focuses on three indicators to predict relapse of NSCLC: SCC antigen concentration, the percentage of lymphocytes with the CXCR2 receptor, and the percentage of monocytes with the CD44v6 receptor. The utility of these indicators for diagnosing and predicting disease-free survival based on preoperative assessment has been previously established. In addition, the advantage of using a combined model that incorporates these parameters has been demonstrated[6].

Studies investigating cytokine receptors and the adhesion receptor CD44v6 in blood cells of NSCLC patients have not been previously carried out; however, existing knowledge indicates that their concentrations increase in the tumor microenvironment[9,10,12-15]. These receptors either result directly from tumor cell metabolism (as in the case of SCC antigen) or accompany the inflammatory process within the tumor and adjacent tissues. Our previous research established that all studied parameters (SCC antigen, CXCR2, CD44v6) reflect the status of proteins involved in tumor growth, with increased concentrations in the blood being characteristic of developing tumors[6]. Therefore, a natural assumption would be a reduction in their levels post-operation, a phenomenon observed 3 weeks after tumor resection in patients with NSCLC. Other researchers measured CYFRA 21-1 and carcinoembryonic antigen (CEA) concentrations 1-month post-tumor resection in patients with NSCLC, reaching similar conclusions[16-20].

It has been noted that the concentration of these biomarkers in the blood of patients with a resected tumor may initially decrease, only to subsequently increase in some cases, a trend that aligns with the dynamics of relapse development[21-24]. Comparative analysis of results from various studies revealed that predicting post-operative relapse based on monitoring CEA concentration in the blood serum yields a sensitivity of 74.7% and a specificity of 69.8%[21-24]. For CYFRA 21-1, the sensitivity and specificity of response were 79.1% and 60.6%, respectively[24].

In the present study, the analysis included several sequential stages: (i) Measuring the level of indicators at different times post-surgery; (ii) calculating the difference in the level of measured parameters in 3-time intervals up to 6 months post-surgery; (iii) analyzing the relationship between changes in the level of measured parameters during these time intervals and the development of relapse; (iv) establishing boundary values of parameters; and (v) assessing the prognostic significance of the determination results for predicting the likelihood of relapse in patients with NSCLC after treatment.

Progressing through these stages, we demonstrated that a significant postoperative increase in the level of

measured parameters is observed exclusively in patients who experience tumor recurrence within a year. In patients without relapse, a slight increase in the level of one or another measured indicator occurred in some cases, but it did not exceed the TV. Other studies have reported an increase in CYFRA 21-1 concentrations in the serum of patients with NSCLC who experienced tumor recurrence within 1 year[17]. Moreover, according to another study, among five serum tumor markers (CYFRA 21-1, CEA, neuron-specific enolase [NSE], carbohydrate antigen [CA] 125, and CA 19-9), only CYFRA 21-1 emerged as the most sensitive marker for predicting response to chemotherapy. An increase in its level after an initial decrease correlated with a high likelihood of tumor relapse[24]. However, the basis for this conclusion relied solely on the results of ROC analysis without calculating the TV and indicators of prognostic accuracy. The study presented only the area under the curve as an argument, which can indicate the quality of the model without the practical applicability.

The utilization of SCC antigen levels in blood serum has been proposed for monitoring treatment efficiency in patients with NSCLC[25]. Elevated concentrations of SCC antigen post-treatment have been demonstrated to be associated with lower overall survival in patients. Furthermore, an increased postoperative SCC antigen level has been recommended as a prognostic marker for recurrence in various cancers, including oral SCC[26], cervical SCC[27], and head-and-neck SCC[28].

In our study, we carried out post-operative monitoring of SCC antigen levels, along with the percentage of lymphocytes with the CXCR2 receptor and monocytes with the CD44v6 receptor in the blood cell populations. This monitoring revealed characteristic dynamics of change at different times after the radical removal of tumors in Stages I–II of NSCLC. Elevated levels before the operation sharply decreased afterward, and a subsequent significant increase in marker concentrations typically indicated the development of relapse. Surprisingly, the prognostic value of these markers, even when considered individually, significantly exceeded the accuracy of predicting the likelihood of tumor recurrence based on the determination of CYFRA 21-1 and CEA.

In the final phase of the study, we achieved a further enhancement in the prognostic accuracy by incorporating all the aforementioned indicators into regression analysis. As a result, their combination was selected for the regression model, wherein, devoid of correlation with each other, they demonstrated the highest predictive capability. This collective predictive ability significantly exceeded that of each individual indicator included separately in the model. The sensitivity of the combined model for a TV of 0.195 was 100%, the NPV was 100%, and the overall accuracy was 96.5%. The AUC, at 0.928, characterizes the model as highly effective. The findings imply that if the TV surpasses 0.195, then in 85.7% of cases, the patient has a genuinely high probability of tumor recurrence, while a TV < 0.195 will correctly predict the recurrence in 100% of patients.

It is essential to highlight that the idea of combining individual criteria into a unified complex to improve their prognostic value is not new. This approach is frequently employed in the laboratory diagnosis of tumors and, in certain cases, serves as an important improving tool[23,29-33]. For example, in predicting relapse for patients with lung AC post-resection, CYFRA 21-1 and human epididymal protein, HE4 exhibited a sensitivity of 80% and a specificity of 57.1% for each marker. However, when both markers were collectively considered, the test specificity increased to 69.7%[23]. In this study, the combined determination of SCC antigen levels, along with the percentage of lymphocytes with the CXCR2 receptor and monocytes with the CD44v6 receptor in the blood cell populations, allowed us to achieve a sensitivity of 100% and a specificity of 95.6%. In practical terms, if the value of the combined model falls below the TV, 95.6% of cases will indeed be free from relapse.

It is crucial to acknowledge that the present study was conducted with a cohort of 57 patients. To validate the proposed model in the future, it is imperative to expand the number of subjects and test its validity on samples.

5. Conclusion

In this study, we observed that the levels of SCC antigen, the percentage of lymphocytes with the CXCR2 receptor, and the percentage of monocytes with the CD44v6 receptor in blood cell populations are highly informative for determining the likelihood of post-operative relapse during the 3 weeks – 6 months after tumor resection. The accuracies are 89.5%, 86.0%, and 82.5%, respectively. By employing a combined model, we could elevate the precision of predicting the probability of relapse based on the measurements of the aforementioned indicators during the 3 weeks – 6 months post-surgery to 96.5% (with a sensitivity of 100% and a specificity of 95.6%, using a TV of 0.195).

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Conflict of interest

All authors declare no conflict of interest.

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Ethics approval and consent to participate

All patients gave written voluntary consent to participate in the study. The study was approved by the decision of the Biomedical Ethics Committee of the Belarusian State Medical University (Committee meeting No. 2, dated 10/04/2021).

Consent for publication

All patients gave written voluntary consent for publishing their data in this paper.

Availability of data

Data used in this work are available from the corresponding author on reasonable request.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Quantitative proteomic analysis reveals regulatory networks of extracellular matrix receptor interaction pathways in endothelial cells after myocardial infarction

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Abstract

Cardiac fibrosis, a significant pathological alteration following myocardial infarction (MI), remains enigmatic with respect to the role of cardiac endothelial cells (ECs). To elucidate the proteomic shifts in cardiac ECs accompanying MI-induced cardiac fibrosis, a standard MI mice model was established through ligation of the left anterior descending branch. Following 14 days of effective modeling, we isolated primary ECs from the hearts of both sham and MI models utilizing the CD31 microbeads sorting technique. Quantitative proteomics and bioinformatics methodologies, including tandem mass spectrometry, were employed to discern proteomic alterations in the primary endothelial cells of the experimental groups. Comprehensive analyses, including Gene Ontology analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, functional enrichment analysis, and functional enrichment cluster analysis, revealed an up-regulation of proteins associated with extracellular matrix-receptor interaction pathway in cardiac fibrosis post-MI. Subsequent Western blot analysis confirmed the up-regulation of specific proteins involved in this pathway, namely collagen type VI alpha 2 (Col6α2), vitronectin (Vtn), and integrin beta (Itgβ). We conclude that the expression levels of Col6α2, Vtn, and Itgβ in primary ECs during the early stage of cardiac fibrosis, 14 days post-MI, were significantly elevated compared to the sham group ($P < 0.05$). This observation suggests that ECM-receptor interaction could potentially influence the progression of cardiac fibrosis following MI.

Keywords: Myocardial infarction; Cardiac fibrosis; Endothelial cells; Proteomics; Extracellular matrix receptor

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1. Introduction

The current landscape sees coronary heart disease as a predominant clinical manifestation of cardiovascular disease. Acute myocardial infarction (MI) poses a substantial risk as a secondary disease, with the potential for dire consequences, including direct

fatality[1]. Advances in MI treatment have resulted in a decline in early mortality rates among patients. However, the impact of MI-induced cardiac fibrosis on patients' quality of life and long-term prognosis remains profound, emerging as a crucial factor in cardiac remodeling and eventual progression to heart failure[2]. The fibrotic process subsequent to MI unfolds in two stages. Early replacement fibrosis typically transpires within 1 – 2 weeks after MI, characterized by the excessive deposition of extracellular matrix (ECM) following myocardial ischemia and necrosis. This fibrotic process leads to the formation of scar tissue, serving as a protective shield for the heart's integrity and averting the risk of cardiac rupture[3,4]. In the majority of cases, the necrosis and loss of myocardial cells do not precipitate heart failure directly. Instead, the occurrence of reactive fibrosis during the middle and late stages emerges as the primary factor contributing to diminished cardiac compliance, ventricular wall stiffness, cardiac dysfunction, and, ultimately, heart failure[5,6]. The complexity of the fibrosis mechanism, coupled with its contrasting early protective effects and long-term development, poses significant challenges in devising effective treatment strategies[5].

At present, the predominant focus of research on the pathological progression and treatment of cardiac fibrosis post-MI focuses on the involvement of immune cells, fibroblasts, and myofibroblasts in the development of cardiac fibrosis. Activated fibroblasts, cardiomyocytes, and diverse immune cells release cytokines to polarize resident macrophages and release chemokines to recruit additional monocytes from the circulation[7,8]. Notably, various fibroblast factors, such as transforming growth factor-β, Ang II, and ET-1, have been identified as promoters of the activation and proliferation of cardiac fibroblasts and myofibroblasts, thereby leading to cardiac fibrosis[9,10]. This process involves neurohumoral factors, the inflammatory response, and oxidative stress, each assuming an important role[11]. Recent studies have revealed that endothelial cells (ECs) also contribute to cardiac fibrosis by secreting these factors. In addition, ECs can undergo endothelial-mesenchymal transition, transforming into stromal cells and subsequently transforming into fibroblasts, thereby exacerbating the development of cardiac fibrosis. However, the specific mechanism governing this transformation remains elusive. A recent study has demonstrated that a majority of fibroblasts involved in the fibrotic process originate from ECs[12]. Consequently, further investigation into the role of ECs in cardiac fibrosis post-MI is warranted.

Previous studies on ECs have mainly used cultured endothelial cell lines to investigate changes in protein expression and phenotypic variations. However, cells cultured *in vitro* may not faithfully represent the authentic state of cells amidst pathological changes. In contrast, primary cells derived from animal models or patient samples offer a more faithful depiction of changes in cell state, cell phenotype, and specific protein expression during pathological processes. However, the acquisition of primary cells presents challenges, requiring larger sample sizes and more complex procedures. These challenges pose a critical hurdle in the current study. Fortunately, this study successfully isolated primary ECs from mouse hearts, presenting valuable real-time samples for disease research. The MI model was established by ligating the left anterior descending branch of the coronary artery in mice, resulting in reactive fibrosis evident 14 days post-MI. Following successful modeling, primary ECs were isolated from mouse hearts for proteomic comparative analysis. This analysis aimed to explore the differences in cell phenotype and protein expression between the MI group and the sham group, identify potential signaling pathways impacting cardiac fibrosis, validate the findings, and assess whether targeted interventions in these pathways could reverse the fibrotic process. These findings may serve as a plausible target for future treatment strategies.

2. Materials and methods

2.1. Establishment of an MI model, induction of cardiac fibrosis, and isolation and purity validation of primary cardiac ECs

Male C57 mice, aged 8 weeks and weighing between 20 g and 25 g, were procured from Weitong Lihua Experimental Animal Center and maintained under SPF-grade animal care conditions. An MI model was created by ligating the left anterior descending coronary artery in the mice. Following intubation, the mice were connected to a ventilator, and a thoracic incision was made to expose their hearts. The bottom edge of the left atrial appendage was ligated using an 8-0 suture thread. The mice were randomly allocated to two groups – the sham operation group and the MI group – each consisting of 30 members. The sham operation group underwent identical surgical procedures as the MI group, excluding the ligation of the left anterior descending artery. Twenty-four hours post-surgery, six mice from each group were euthanized under deep anesthesia and administered ice-cold PBS by gavage. Heart sections were then obtained and incubated with a 1 – 2% 2,3,5-triphenyl-tetrazolium chloride (TTC) solution at 37℃ for 20 min. Fourteen days following the MI, paraffinized heart sections from six mice in each group were stained using Sirius Red. The remaining mice were dissected into pieces and treated with collagenase type II (prepared in Hank's solution, concentration 1 – 2 mg/ml, at 37℃ for 30 – 45 min). Primary cardiac endothelial cells were subsequently isolated using CD31 magnetic

microbeads. A portion of the isolated cells were labeled with CD31-PE, followed by a flow cytometric examination to determine the concentration of ECs. The remaining cells were cryogenically preserved in a liquid-nitrogen bath and subsequently transferred to an −80℃ storage unit.

2.2. Tandem mass tag-proteome sample preparation, high-performance liquid chromatography sorting, and mass spectrometry detection

Protein lysate containing 1% TritonX-100 and 1% protease inhibitor was added to the sample. Following a 4-h incubation, the mixture was centrifuged at 12,000 rpm for 10 min, after which the cell debris was removed. The supernatant, harboring the extracted protein, was then transferred to a new Eppendorf tube, and the protein concentration was quantified using a Bicinchoninic acid protein assay kit. Based on the concentration determination, equivalent protein amounts were selected, and the sample volume was adjusted to 500 μL with 8M urea. Subsequently, a 20% trichloroacetic acid (TCA) was added and incubated in ice water for 2 h or overnight. After centrifugation at 4℃ for 5 min, the supernatant was discarded, and the pellet was washed three times with ice-cold acetone. The protein precipitate was air-dried, followed by the addition of 200 mm tetraethylammonium bromide (TEAB) and dispersion by ultrasound. Trypsin, at a ratio of 1:50 (protease: protein, M/M), was then added for overnight hydrolysis at 37℃. Following this enzymatic digestion, 5 mm dithiothreitol (DTT) was added to achieve a final concentration and reduced at 37℃ for 1 hour. A final concentration of 15 mm iodoacetamide (IAM) was added and left in the dark at room temperature for 45 min.

The trypsin-hydrolyzed peptides were then desalted with Strata X C18 (Phenomenex) and lyophilized. The peptides were reconstituted with 0.5 M TEAB and labeled according to the instructions provided by the tandem mass tagging (TMT) kit (label reagents for sham-1, sham-2, sham-3, MI-1, MI-2, and MI-3 were 126, 127, 128, 129, 130, and 131, respectively). The labeling procedure involved dissolving the thawed reagents in acetonitrile, mixing them with the peptides, incubating them at room temperature for 2 h, and subsequent desalting and freeze-drying.

Next, the peptides were fractionated using high-pH reverse-phase high-performance liquid chromatography (HPLC) on an Agilent 300 Extend C18 column (5 μm diameter, 6 mm inner diameter, 250 mm length). Elution occurred with a gradient of 8 – 32% acetonitrile, pH 9, over 60 min, resulting in 60 fractions that were subsequently combined into 14, followed by lyophilization for the subsequent steps. Finally, the polypeptides were dissolved in the mobile phase A of a liquid chromatography system and separated using an EASY-nLC1000 ultra-performance liquid chromatography system.

The separated polypeptides were ionized via an NSI ion source and were subsequently analyzed using Orbitrap Fusion mass spectrometry. The ion source voltage was set at 2.0 kV, facilitating the detection and analysis of both the parent ion and secondary fragments of the polypeptides by a high-resolution Orbitrap. The scan range was set from 400 – 1500 m/z, and the resolution was set to 60000. For secondary mass spectrometry, the scanning range was set to 100 m/z, with a resolution of 15000. Data acquisition was performed using data-dependent acquisition settings, which sequentially scanned the top 20 peptide parent ions with the most substantial signal intensity into a higher-energy collisional dissociation collision cell pool. The fragmentation energy was set to 32%, and secondary mass spectrometry was carried out sequentially. To enhance mass spectrometry (MS) utilization, automatic gain control was set at 5e4, the signal threshold was set at 20000 ions/s, the maximum injection time was set at 70 ms, and dynamic exclusion was employed for 30 s to prevent redundant scanning of parent ions.

2.3. Data analysis

Infarct size, determined through TTC staining, and collagen fiber area, assessed through Sirius Red staining, was quantified using ImageJ. Statistical analyses were performed using Prism 7.0, and statistical significance was determined through Student's *t*-test. The resulting data were presented with a 95% confidence interval, where $P < 0.05$ signified statistical significance.

Secondary MS data were acquired using Maxquant (v1.5.2.8) with the following parameters: the Mus_musulus_10090_SP_20191115 (17,032 sequences) was employed, supplemented with a reverse library for false positive rate calculation due to random matching. A common contaminated library was included to eliminate the potential impacts of protein contamination. The digestion mode employed trypsin/P was the digestive mode, allowing for a maximum of 2 missing cleavage sites and a minimum peptide length of 7 amino acids. Mass error tolerance rates for primary and secondary ions in the first and main searches were set at 20 ppm and 5 ppm, respectively, with the secondary fragment ion set at 0.02 Da.

The fixed modification involved the alkylation of cysteine, and variable modifications included methionine oxidation, N-terminal acetylation, and deamidation (NQ). The quantification method was conducted using a TMT-6plex kit, and both protein identification and

peptide spectrum match identification adhered to a 1% false discovery rate.

2.4. Data normalization

Our protein normalization methodology relies on the fundamental assumption that the sum of intensities across six tags in the six-complex system remains constant. Following normalization, we evaluated the relative abundance of MI and sham in our experiment by calculating the ratio of average reporter ion intensities to trypsin peptide reactions. The resulting expression ratios for MI/sham or sham/MI in three pairs of repeat samples constituted our three-paired sample analysis. We set a 1.5-fold change as our threshold for differential expression, indicating that the ratio of MI/sham or sham/MI surpasses 1.5. Statistical significance for observed protein alterations was assessed using the Student's *t*-test.

2.5. Bio-informatics analysis

Utilizing the UniProt-GOA database, we categorized proteins through Gene Ontology (GO) analysis, considering cellular components, molecular functions, or biological processes. To explore functional enrichment, we employed the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, organizing pathways via the KEGG hierarchical classification method. The InterPro database was used to investigate various functional domains of differentially expressed proteins. Fisher's exact test assessed the enrichment level for the aforementioned protein functions, with $P < 0.05$ indicating significant differences. To unravel potential interrelationships and discrepancies among proteins within a specific KEGG pathway, cluster analysis based on functional enrichment was employed. We collected functional classification data and associated enrichment *P*-values, screening for significant enrichment ($P < 0.05$) across at least one proteome. The *P*-value data matrix, converted using log10, was classified through Z-transform. For the exploration of protein-protein interaction networks, we utilized the string database (v.10.5). Interactions with a confidence level exceeding 0.7 were extracted and visualized using the R software package, networkD3.

3. Results

3.1. The establishment of MI model

After ligating the left anterior descending coronary artery for 24 h, the mice were euthanized under deep anesthesia. Following perfusion with ice-cold PBS, the mouse heart was isolated and preserved. The non-infarction area (red area) and infarct area (white area) were distinguished through TTC staining. Infarct size was semi-quantitatively compared by calculating the percentage of infarct size to the left ventricular area (IS/LV%). The results indicated a significantly larger infarct size in the MI group compared to the sham operation group, as depicted in Figure 1A. After 14 days of MI, heart samples were collected, and paraffin sections were prepared. Sirius Red staining revealed collagen deposition (stained in red color) in the heart, observed and photographed using a light microscope. The collagen area was quantified using ImageJ software, and the collagen volume fraction (CVF) was calculated. Sirius Red staining demonstrated a notably higher level of collagen deposition in the MI group compared to the sham group, as illustrated in Figure 1B. The corresponding statistical results are presented in Figure 1C, showing IS/LV% and CVF% data. In summary, the MI model was successfully established, and evident cardiac fibrosis was observed after 14 days, as evidenced by the results of infarct size analysis and collagen deposition assessment.

3.2. Isolation of primary cardiac ECs and quantitative proteomic analysis of TMT

After 14 days, the mice were euthanized under anesthesia. Heart samples were dissected and subjected to digestion. The isolated ECs were sorted using CD31 magnetic microbeads. Flow cytometry was employed to assess the purity of ECs. The remaining cells underwent protein extraction, trypsin hydrolysis, and TMT labeling and were subsequently analyzed by mass spectrometry. The detailed process is illustrated in Figure 2A. The results revealed that the proportion of ECs was 88.96 ± 0.48% ($n = 3$) (Figure 2B). Employing a 1.5-fold change as the threshold for protein differential expression, we identified 395 proteins in both the MI and sham groups. Among these, 161 proteins exhibited up-regulation, while 234 proteins displayed down-regulation ($P < 0.05$) (Figure 2C). A differential protein volcano map, plotting the logarithm of log2 as the abscissa and the logarithm of -log10 as the ordinate, was generated. In the figure, red dots signify up-regulated proteins with significantly different expression, while blue dots represent down-regulated proteins with significantly different expression (Figure 2D).

3.3. Go analysis of differential proteins

We classified the differential proteins into GO subclasses and presented them in terms of biological processes, cellular components, and molecular functions (Figure 3). In the biological process category, 122 proteins were up-regulated in cellular processes, 105 in single-organism processes, and 101 in biological regulation. Conversely, the down-regulated proteins encompassed 189 proteins in single-organism processes, 185 in cellular processes, and 161 in metabolic processes. Within the cellular component classification, 151 proteins in cell components

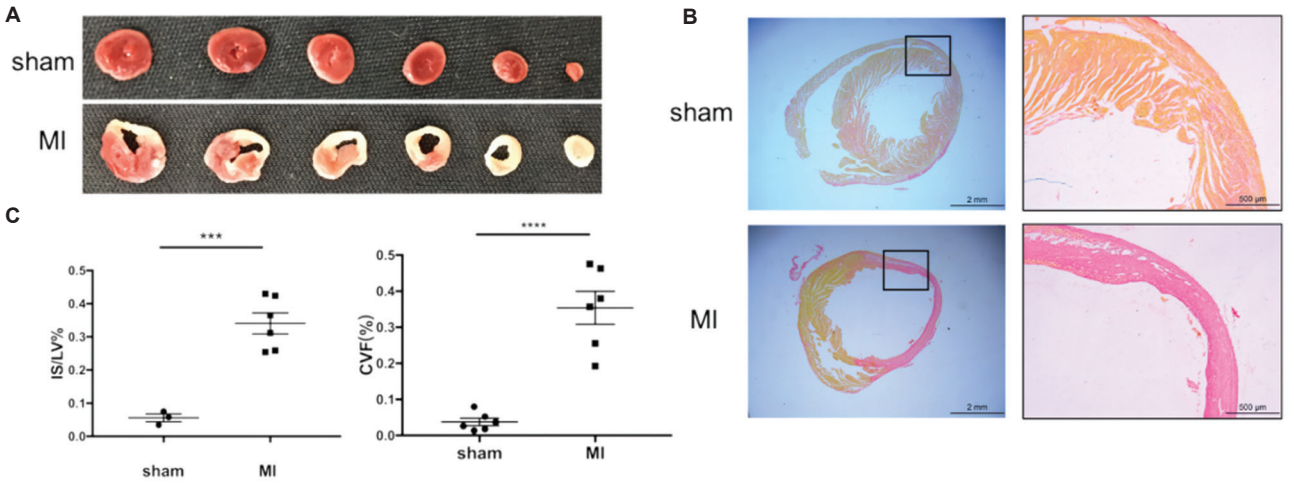

Figure 1. The myocardial infarction model was successfully established. (A) The representative map shows triphenyl tetrazolium chloride staining of heart sections, with red indicating the non-infarction area and white indicating the infarcted area. (B) Representative images of collagen deposition areas stained with Sirius Red, where red represents the collagen deposition area. (C) Statistical chart for IS/LV% (34.06 ± 3.19% [$n = 6$] vs. 5.59 ± 1.19% [$n = 3$]; $P < 0.05$) and CVF% (39.8 ± 7.16% vs. 2.78 ± 1.23%, $n = 6$, $P < 0.05$).

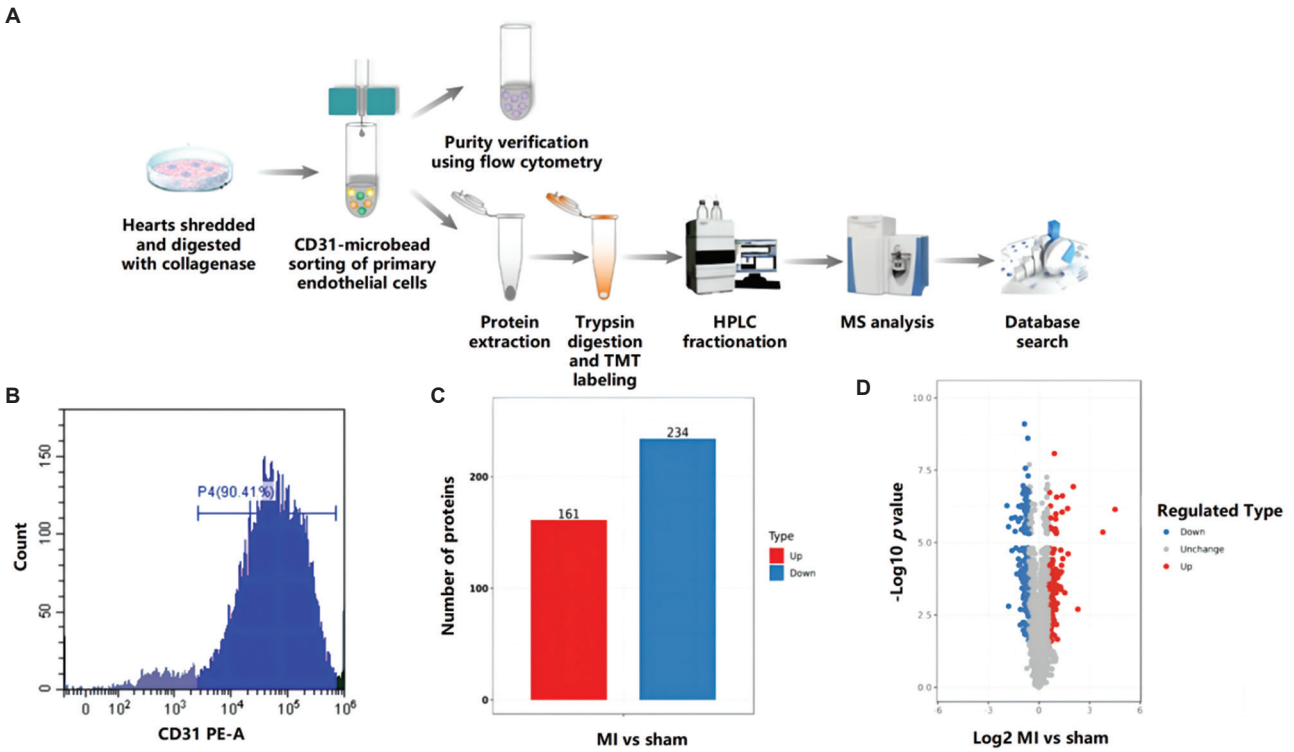

Figure 2. TMT proteomics detection of primary endothelial cells. (A) Flow chart of the methodology. (B) Verification of endothelial cell purity by flow cytometry. (C) Overview of differential protein detection by TMT proteomics. (D) Differential protein volcanic map.
Abbreviations: HPLC: High-performance liquid chromatography; MS: Mass spectrometry; TMT: Tandem mass tagging.

were upregulated, 138 in organelle components, and 85 in membrane components. Conversely, the down-regulated proteins involved 228 proteins in cell components, 224 in organelle components, and 116 in membrane components. In terms of molecular functions, the up-regulated proteins mainly exhibited binding functions, involving 131 proteins, and catalytic activity function, with 43 proteins. On the other hand, the down-regulated proteins were mainly associated with binding function, involving 157 proteins, and catalytic activity, involving 141 proteins.

3.4. KEGG analysis of differential proteins

We analyzed the KEGG function of different proteins. The results, as illustrated in Figure 4A, highlight that the main KEGG pathways associated with up-regulated proteins include DNA replication, complement and coagulation cascade, cell cycle, and ECM-receptor interaction. Conversely, Figure 4B illustrates that KEGG pathways enriched with down-regulated proteins encompass the TCA cycle, oxidative stress, Parkinson's disease, Huntington's disease, Alzheimer's disease, and thermogenesis.

3.5. Protein–protein interaction analysis of differential proteins

We selected differential proteins for protein-protein interaction analysis. The proteins with interaction relationships among the differential proteins are depicted in Figure 5. In the ECM-receptor interaction pathway,

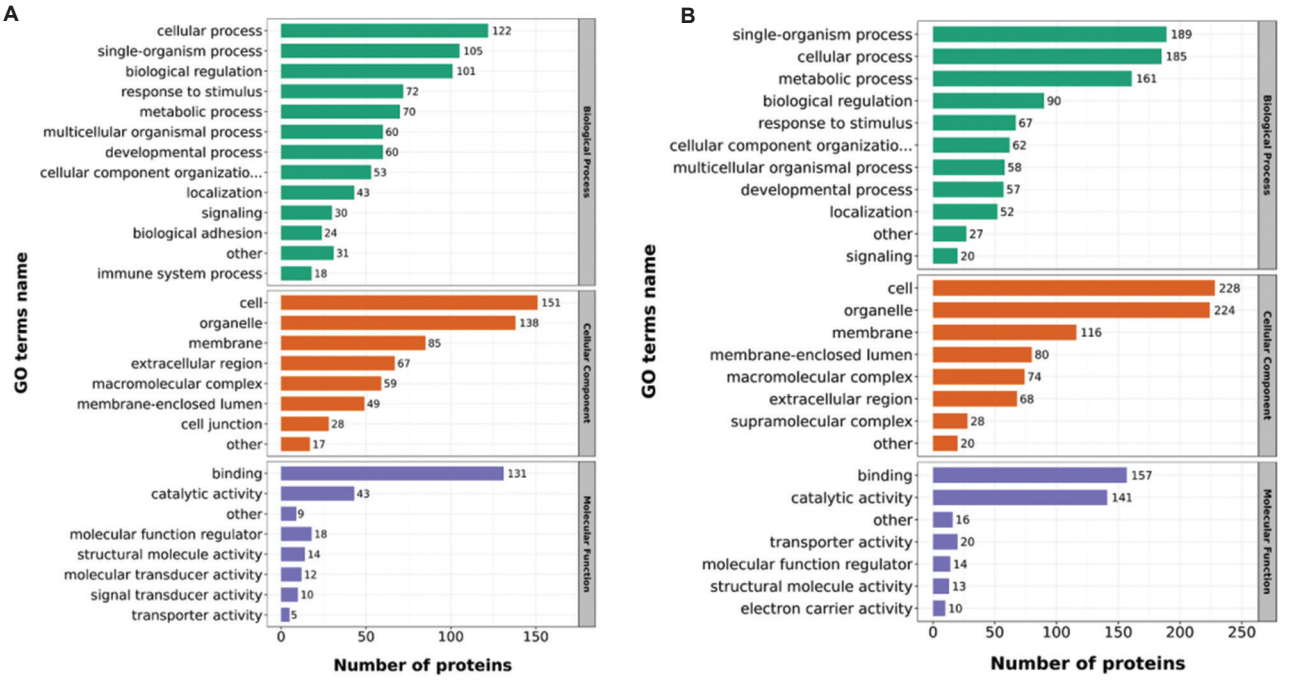

Figure 3. Gene Ontology (GO) analysis of differential proteins. (A) GO analysis of up-regulated proteins. (B) GO analysis of down-regulated proteins.

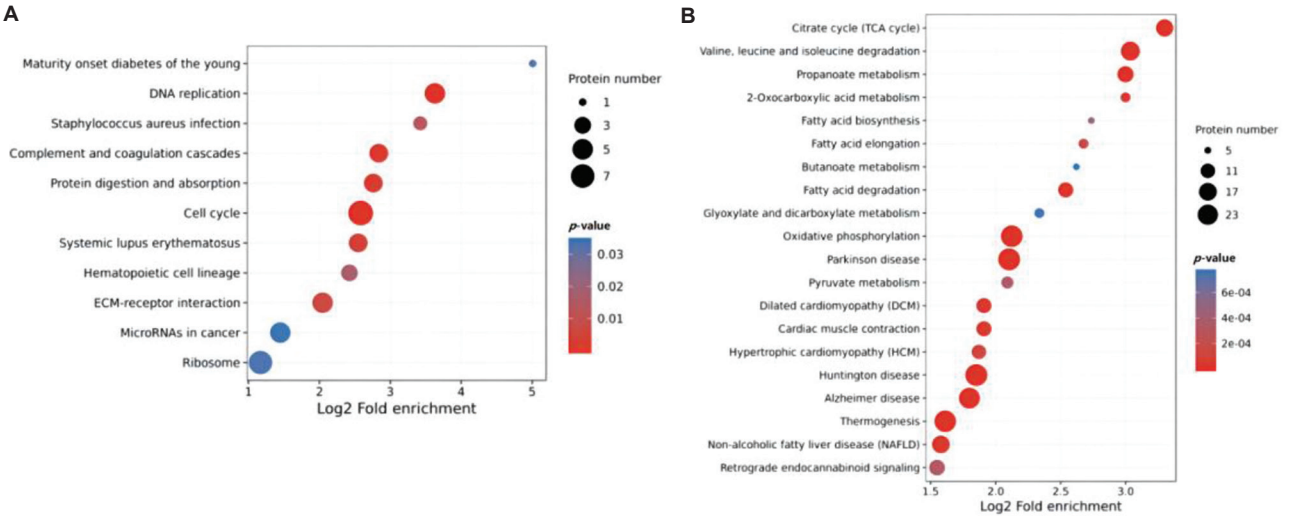

Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differential proteins. (A) KEGG analysis of up-regulated proteins. (B) KEGG analysis of down-regulated proteins.

collagen type VI alpha 2 (Col6α2), collagen type VI alpha 1 (Col6α1), vitronectin (Vtn), integrin alpha 2 beta (Itgα2b), and integrin beta 3 (Itgβ3) are up-regulated, and there is protein interaction observed among them.

3.6. Cluster analysis of differential proteins, cluster analysis based on the KEGG pathway, and screening of target pathway

Cluster analysis was performed on the genes corresponding to the top 50 differentially expressed proteins, and the resulting heatmap is presented in Figure 6A. Based on the differential expression fold, we divided them into four quartiles: Q1 comprised 26 proteins with a differential expression fold of less than 0.5; Q2 included 208 proteins with differential expression folds between 0.5 and 0.667; Q3 contained 129 proteins with differential expression folds between 1.5 and 2.0, and Q4 comprised 32 proteins with differential expression folds above 2.0. Each Q group was subjected to KEGG pathway enrichment analysis, and cluster analysis was performed to reflect the correlation between up-regulated or down-regulated proteins and differential expression folds in related functional pathways, providing intuitive evidence for identifying target pathways (Figure 6B). Our focus narrowed to the up-regulated or down-regulated proteins in the ECM-receptor interaction pathway of interest (Figure 7). This targeted exploration sets the stage for the subsequent step of screening related target proteins.

3.7. Selection of ECM-receptor interaction as the target pathway to verify the up-regulation of protein expression

Through KEGG enrichment analysis of up-regulated proteins, we observed significant enrichment in the

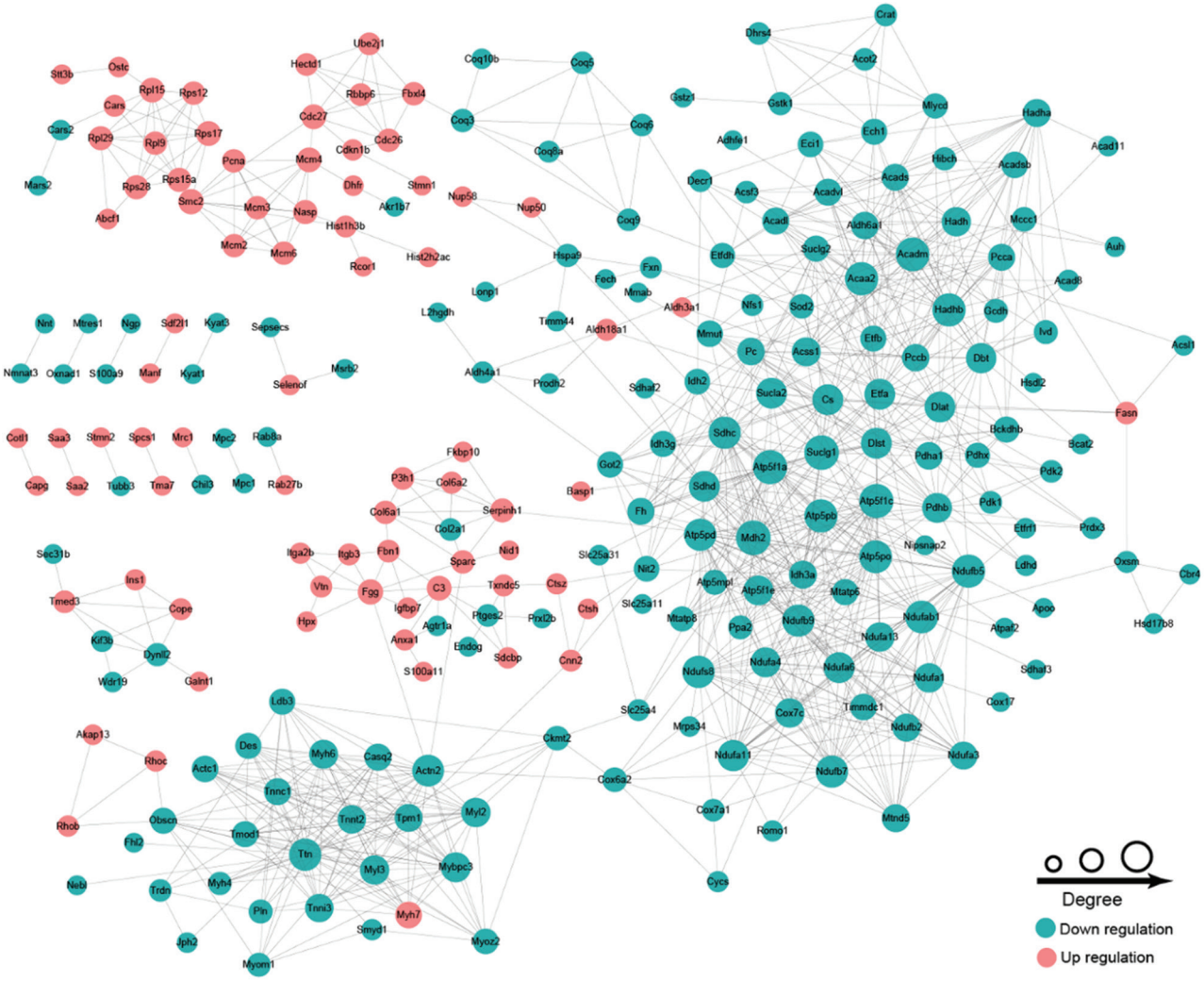

Figure 5. Protein-protein interaction analysis of differential proteins.

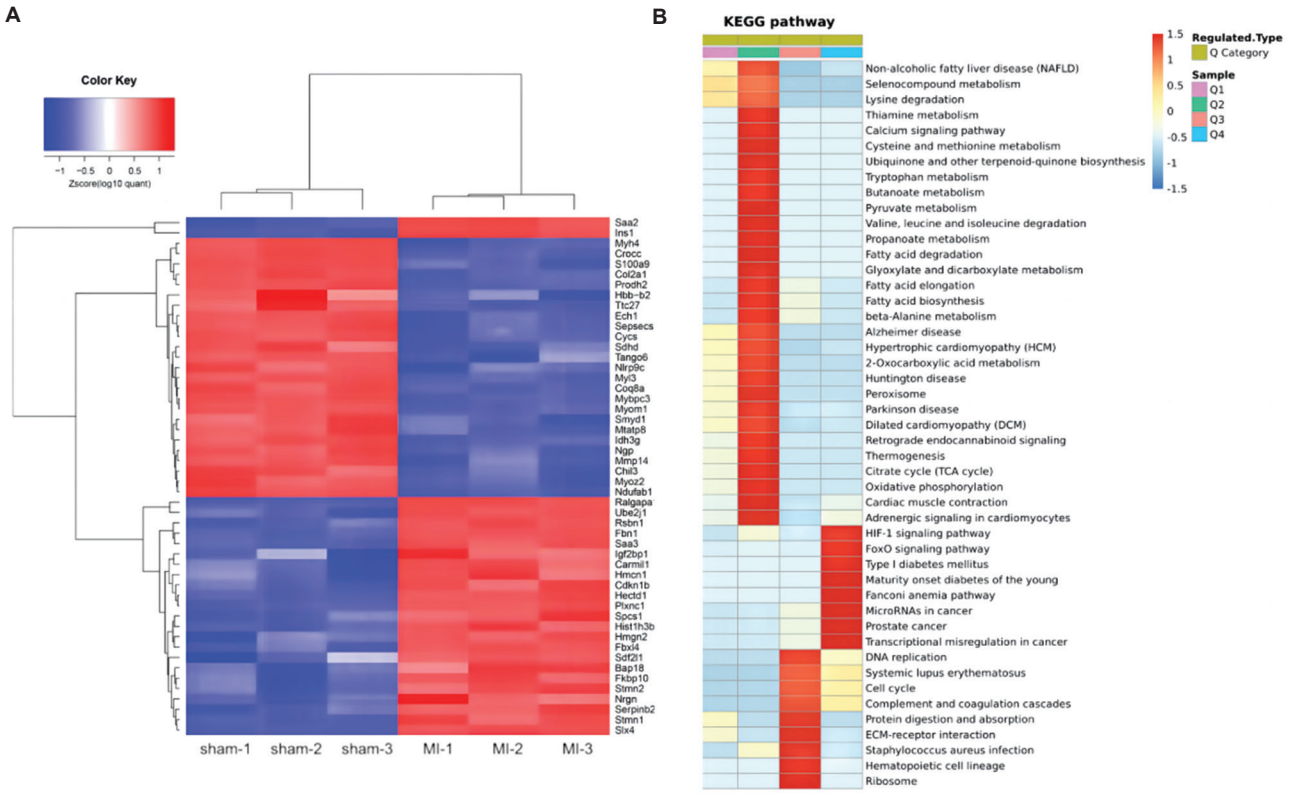

Figure 6. Heatmap of cluster analysis. (A) Heatmap of the first 50 genes with different expressions. (B) Heat map of cluster analysis based on Kyoto Encyclopedia of Genes and Genomes pathway enrichment.

ECM-receptor interaction pathway. In this study, the up-regulated proteins identified include Col6α2, Col6α1, Vtn, Itgα2b, and Itgβ3, with their corresponding genes and up-regulated folds detailed in Table 1. Western blot analysis further validated the expression levels of the aforementioned proteins (Col6α2, Vtn, Itgβ3), demonstrating that their expression in the MI group was significantly higher than that in the sham group. The differences in protein expression levels are detailed in Figure 8. The expression of target pathway-related proteins was significantly up-regulated ($P < 0.05$). In conclusion, we postulate that the upregulation of Col6α2, Vtn, and Itgβ3 after MI may contribute to the occurrence and development of cardiac fibrosis through the ECM-receptor interaction pathway. Further verification is required to elucidate the specific mechanism.

4. Discussion

This study aimed to investigate the mechanism of ECs in cardiac fibrosis after MI. The comprehensive schematic of the study is depicted in Figure 9. We established the MI animal model by ligating the left anterior descending coronary artery of mice, confirmed by TTC staining (Figure 1A). To simulate cardiac fibrosis post-MI, we calculated CVF through Sirius Red staining to assess myocardial collagen deposition. The results illustrated evident collagen deposition and the appearance of cardiac fibrosis in the MI group (Figure 1B). Given that collagen deposition is a clear indicator of reactive fibrosis in heart tissue, we selected this specific time point to isolate primary ECs from the heart for further comparative analysis.

The successful extraction of primary cardiac ECs is a pivotal step in this study. Primary mouse cardiac ECs were isolated using CD31 magnetic microbeads. While existing literature has applied this method to isolate primary cardiac ECs from the lungs and kidneys of mice, its application to isolate ECs from the heart post-MI presents challenges due to their low content. Ensuring the accuracy of subsequent proteome detection necessitates maximal purification of the extracted cells. Leveraging CD31 as a specific EC marker, the isolated cells were fluorescently labeled with CD31-PE. Flow cytometry analysis demonstrated that the purity of ECs in the samples for proteomic analysis could achieve approximately 89% (Figure 2B). To guarantee the provision of protein of 100 μg from a single sample, primary ECs from 6 – 8 mouse hearts were isolated for each sample. Subsequent to mass spectrometry, the data underwent bioinformatics analysis.

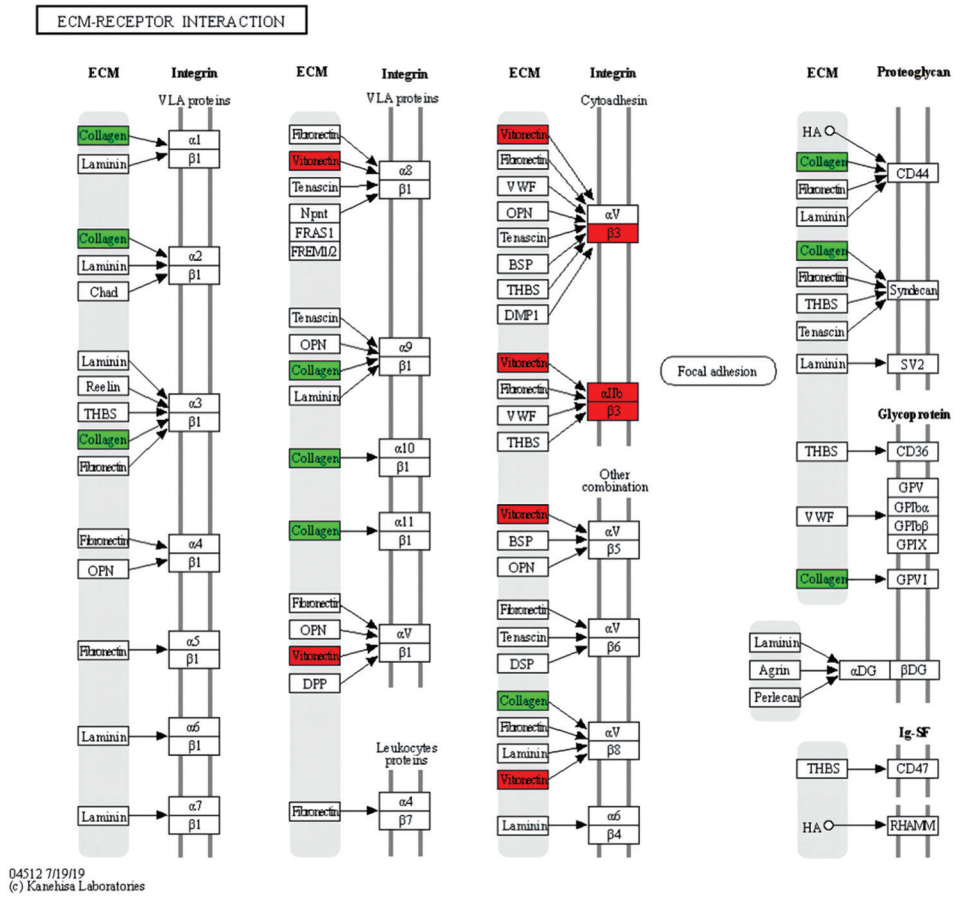

Figure 7. Schematic diagram of extracellular matrix receptor interaction pathway with significant enrichment of differentially up-regulated proteins. Red indicates up-regulated proteins, and green indicates down-regulated proteins.

Table 1. Up-regulated proteins in the ECM-receptor interaction

Pathway	Gene	Protein names	Relative ratio
ECM-receptor interaction	Col6α2	Collagen alpha-2(VI) chain	1.73
	Itga2b	Integrin alpha-IIb	1.77
	Vtn	Vitronectin	1.92
	Col6α1	Collagen alpha-1(VI) chain	1.76
	Itgβ3	Integrin beta-3	1.57

Abbreviation: ECM: Extracellular matrix.

After 14 days' post-acute MI, the heart progressively underwent fibrotic changes. Primary ECs from the mouse heart were isolated for TMT-based quantitative proteomic analysis. A significant difference in protein expression emerged between the MI and sham groups. Utilizing GO, KEGG, and functional enrichment cluster analyses, among other bioinformatics tools, we identified a specific focus on the HIF-1 pathway, DNA replication pathway, and ECM-receptor interaction pathway (Figures 5A and B). Proteins within these pathways exhibited both up-regulation and down-regulation. Our attention was drawn to the up-regulated proteins – Col6α2, Col6α1, Vtn, Itga2b, and Itgβ3 – in the ECM-receptor interaction pathway (Table 1). We predict that ECs may promote cardiac fibrosis by up-regulating these proteins post-MI. At present, an increasing number of studies focus on how proteins secreted by ECs interact with organs, affecting the onset and progression of corresponding diseases[13]. Therefore, we find it particularly meaningful to explore how the ECM-receptor interaction pathway affects cardiac

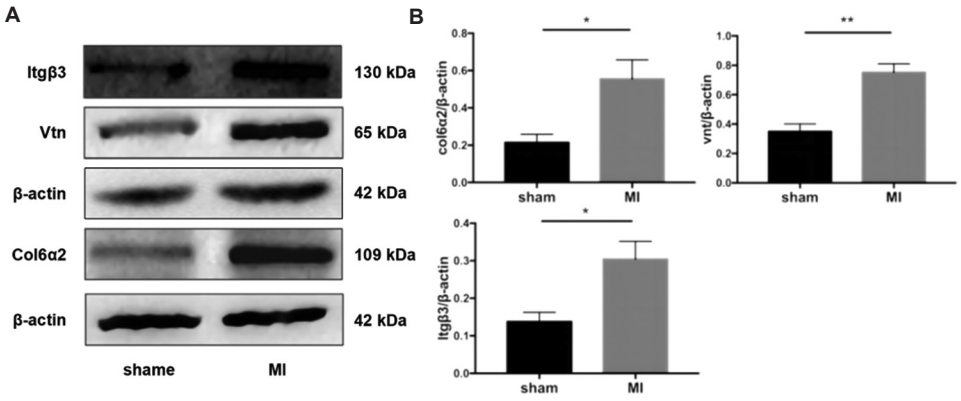

Figure 8. Western blot validation. (A) Western blot representative map. (B) Statistical analysis of the Western blots.

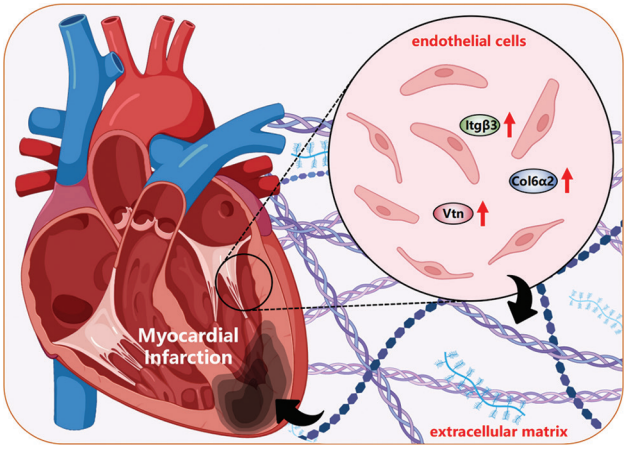

Figure 9. Following a myocardial infarction, there is a widespread alteration in the expression of proteins within the cardiac endothelial cells, with a corresponding elevation in the levels of Itgβ3, Col6α2 and Vtn. These alterations in protein levels are principally associated with the extracellular matrix receptor pathways and exert an influence upon the heart, modulating processes such as cardiac remodeling in the aftermath of the infarction.

fibrosis (Figure 7). Diverging from other endothelial-related studies, this study aims to explore how changes in endothelial protein secretion interact with other cells, thereby affecting the occurrence and development of cardiac fibrosis.

Based on the aforementioned results, the ECM-receptor interaction pathway has emerged as a focal point of investigation. Although extensively studied in various diseases, its exploration in cardiovascular diseases, especially in the context of cardiac fibrosis post-MI, remains limited. In the following discussion, we will provide a brief analysis of the proteins associated with this pathway. Previous studies have highlighted the role of extracellular matrix receptor interaction pathways in cancer, gastric cancer[14], atrial fibrillation[15], diabetic nephropathy[16], and wound healing[17]. However, its role in cardiovascular diseases, specifically cardiac fibrosis post-MI, has been a research gap. In our study, we identified up-regulated proteins in this pathway, including Col6α2, Col6α1, Vtn, Itga2b, and Itgβ3. Verification through Western blot confirmed increased content of Col6α2, Vtn, and Itgβ3 (Figure 8). Col6α2-chain, a constituent of type VI collagen and one of its three components has been identified in various musculoskeletal tissues. However, its functional role in bone has only become a subject of study[18]. The implications of Col6α2 in cardiovascular disease remain a knowledge gap. The potential impact of the ECM-receptor interaction pathway on cardiac fibrosis requires further exploration. Integrin β3, a product of the Itgβ3 gene, is composed of α and β chains, constituting a complete cell surface protein. Integrins play a pivotal role in cell adhesion and cell surface-mediated signal transduction. Operating as transmembrane receptors, they serve as a bridge connecting cells to ECM and, in certain cases, facilitate interactions between cells. Therefore, one of their main functions is to link the ECM with the cytoskeleton. Most importantly, during sustained myocardial contraction, integrins are considered mechanical transducers[19-21]. Their involvement extends to the development of fibrosis across multiple organs, with a particular emphasis on the heart[22]. Vitronectin is present in both serum and tissues, representing a secreted protein. Its signal transduction pathways are hypothesized to be interconnected with those of growth factors, necessitating further exploration of their association with cardiovascular diseases[23].

In this study, we identified three up-regulated target proteins, namely Col6α2, Vtn, and Itgβ3, in primary ECs derived from the mouse heart. To elucidate the specific molecular mechanisms associated with these target proteins, further verification and analysis are imperative. Our further study will involve conducting *in vitro* verification experiments. Specifically, we plan to select human cardiac microvascular ECs for protein

content detection. In addition, RNA interference will be employed to reduce the expression of a targeted protein within the cell line, while overexpression of the target protein will allow us to identify changes in the expression of other related proteins within the target pathway. This approach aims to clarify whether Col6α2, Vtn, and Itgβ3 exert their effects through the ECM-receptor interaction pathway. Furthermore, empirical testing will be conducted to determine whether inhibitors of the target proteins can induce changes in the fibrotic phenotype. This evaluation will involve assessing the degree of fibrosis in an animal model of cardiac fibrosis following MI. In the future, a comprehensive understanding of the interplay between the ECM and ECs during the cardiac fibrosis process will be attained by conducting meticulous analyses of cardiac ECs at various junctures post-MI. This effort will contribute to the construction of a more comprehensive interaction network atlas.

In this study, we isolated primary ECs from MI mice and analyzed their profiles of differential protein expression. Our findings highlight the significant involvement of the ECM receptor pathway in regulating ECs post-MI. However, it is essential to note that the verification of the ECM receptor in the MI mouse model has not been conducted, representing a critical aspect that requires further supplementation and refinement in subsequent research conducted by our group.

5. Conclusion

ECs potentially contribute to cardiac fibrosis post-MI by regulating ECM-receptor interaction. While this study successfully verified the target proteins through Western blot, further validation in both *in vivo* and *in vitro* settings is crucial to unravel underlying mechanisms. The precise mechanism of action remains unclear, forming a key focus for the next phase of experimental planning. In the context of cardiac fibrosis, accurately selecting sampling nodes and understanding their protective or adverse effects on the heart after MI is essential. The definition of time points within different stages of the fibrotic reaction represents a time range, necessitating the examination of multiple time nodes to study the dynamic changes of TMT-related proteins. However, achieving a comparison of multiple time nodes is challenging due to the associated requirement for a large sample size. In our study, we initially selected the 2-week post-MI time node for high-throughput screening of TMT quantitative proteomics. The screening results can guide the subsequent selection of multiple nodes for further verification. The purpose of this study is to identify a molecular pathway capable of inhibiting fibrosis development post-MI without interfering with protective mechanisms. The goal is to explore treatment methods and targets that can alleviate late reactive fibrosis while preserving the early scar repair process.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

The animal ethics of this project have been approved by Peking University Third Clinical Medical School Ethical Committee of Animals (LA2022077).

Consent for publication

Not applicable.

Availability of data

Not applicable.

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ACCSCIENCE PUBLISHING

Global Translational Medicine

CASE REPORT

Management of persistent genital arousal disorder: A case report

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Abstract

Persistent genital arousal disorder (PGAD) is a pathological condition characterized by intrusive, unwanted, and distressing symptoms related to spontaneous and prolonged sensations of genital arousal in the absence of sexual desire or stimulation that may compromise the individual's quality of life. Here, we report the case of a woman who suffered from PGAD probably associated with multiple alterations in the spinal column and Tarlov cyst, which caused the compression of nerve roots and the abnormal sensitivity of the genital region.

Keywords: Orgasm; Sexual arousal; Arousal disorder

1. Background

Persistent genital arousal disorder (PGAD) is a pathological condition characterized by intrusive, unwanted, and distressing symptoms related to spontaneous and prolonged sensations of genital arousal in the absence of sexual desire or stimulation[1]. PGAD causes significant discomfort, compromises the individual's quality of life, and promotes feelings of shame, which can often lead to isolation and suicidal ideation[2].

The criteria for the diagnosis of PGAD are not well defined. Most people who suffer from this condition show several recurrent symptoms. In 2001, Leiblum and Nathan[3] proposed the following diagnostic criteria: (i) Persistently aroused genitalia, (ii) arousal that remains after orgasm or that requires multiple orgasms to reduce, (iii) arousal unrelated to sexual desire, (iv) arousal triggered by sexual and non-sexual stimuli, and (v) intrusive and unwanted symptoms. Unwanted sexual arousal remains the most common symptom among those with PGAD, accounting for 91.3% of the reported symptoms[2]. Further criteria include sensations of being on the verge of orgasm and duration of clinical presentation of at least 6 months[4].

The prevalence of PGAD has been underestimated, as it is approximated from studies with small sample sizes. A joint analysis of both American and Canadian populations, including 1,267 women, 360 men, and seven non-binary individuals, revealed that 1–4% met the criteria for PGAD[5]. The symptoms were more prevalent in the population between 20 and 59 years of age, with the lowest rates reported among those aged 60 and above[5].

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The etiology of PGAD is multifactorial and may be related to vascular, neurological, pharmacological, and psychological alterations[1,6]. Anomalies in the pelvic arteriovenous system, such as pelvic varicose veins and arteriovenous malformations, can lead to changes in blood flow and cause persistent engorgement of the genital region, leading to PGAD[7]. Among the known neurological factors, central and peripheral alterations, nerve entrapment arousal deserve emphasis as a source of continuous arousal[8], however, complementary tests are not predictive of this association[9]. Furthermore, there are still gaps regarding the mechanism of female sexual arousal related to peripheral and spinal nerve pathways and neurotransmitters[10]. Nevertheless, it is possible that expansive lesions or arteriovenous fistulas, as well as post-surgical changes, cerebrovascular accidents, and cervical disc anomalies, result in alterations in the ascending and descending nervous pathways responsible for the autonomic and motor regulation of the pelvic region, leading to the engorgement of the genital area and thereby triggering arousal. There is evidence that definitive treatment of the underlying neurological factor promotes the improvement or resolution of symptoms in up to 80% of cases[10]. Epilepsy is an example in which, after optimization of the anticonvulsant drug regimen, patients showed improvement in the concomitant presentation of PGAD[11].

Tarlov cysts are single or multiple structures that originate from the nerve root, located mainly in the sacral spine[12], and are found in 38% of patients with PGAD[13]. Surgical treatment has been shown to alleviate or eliminate the symptoms[14].

Drug-related causes are particularly associated with the initiation or abrupt discontinuation of selective serotonin reuptake inhibitors (SSRIs)[15]. In addition, psychological factors such as anxiety can exacerbate the presentation of PGAD[16].

Here, we report the clinical management of PGAD in a female patient. To this end, a review of the medical records was carried out to obtain data concerning the patient's first consultation at the Human Sexuality Studies Outpatient Clinic (AESH) of the Center for Human Reproduction of the Department of Gynecology and Obstetrics of the Ribeirão Preto Medical School - University of São Paulo (FMRPUSP). One of the researchers in this study (T.T.M.) approached the woman while she was waiting to be attended at the clinic and explained the characteristics of the case report. After agreeing to participate, the participant signed an informed consent form. This case report was approved by the Research Ethics Committee of the FMRPUSP Clinics Hospital (approval ID: CAAE - 69898023.0.0000.5440).

2. Case presentation

A 50-year-old uniparous, married woman, who worked as a teacher, had her first appointment at the AESH on February 13, 2023, with the main complaint of spontaneous and recurrent orgasms, with around 15 episodes a day. This persistent arousal condition became prominent in April 2022, when she was diagnosed with a disc herniation between L2-S1 and radicular compression of L2-S1. The patient had a history of bilateral pain in the lumbar region, with irradiation initially to the anterior surface of the right leg, then to the left. She also presented genital arousal and constant involuntary orgasms, which worsened when she sat and/or lay down. After undergoing percutaneous denervation on January 5, 2023, she complained of sequelae of high-intensity compressive pain in the coccygeal region, which was triggered by touch. The patient associated the intense distress with the condition and claimed to have had suicidal thoughts. She also reported having undergone two lumbar sympathetic blocks in June 2022 and January 2023 (undocumented), which however did not improve her clinical condition.

The patient suffered from systemic arterial hypertension, fibromyalgia, and type 2 diabetes mellitus. The medications she used include desogestrel 75 μg/day, fluoxetine 60 mg/day, enalapril 10 mg/day, hydrochlorothiazide 25 mg/day, ezetimibe 10 mg/day, alprazolam 0.5 mg/day, trazodone 50 mg/day, pregabalin 150 mg, and amlodipine 10 mg/day. The patient had undergone bariatric surgery in 2016 and percutaneous denervation of the sacral region in 2022 (undocumented).

Genital examination did not reveal anatomical alterations nor abnormal exposure of the clitoris. Her vaginal muscle had adequate tone, with no trigger points for pain, although she claimed that applying pressure on the vaginal wall could relieve the arousal sensation.

Transvaginal ultrasonography in July 2022 showed no abnormality. On April 17, 2023, nuclear magnetic resonance spectroscopy of the patient's spine was conducted, revealing spondylosis, discrete disc alterations, and a cystic image in the vertebral canal at the level of the S2, which was suggestive of a Tarlov cyst. The magnetic resonance imaging (MRI) of the right hip showed insertional tendinopathy and peritendinitis of the gluteus medius and minimus muscles, without ruptures, while the MRI of the left hip revealed insertional tendinopathy and peritendinitis of the gluteus medius and minimus muscles, with trochanteric bursitis.

Bilateral transcutaneous blockage of the pudendal nerve was performed, guided by digital palpation of the ischial spines that can be identified along the vaginal

posterolateral sidewalls, with 10 mL of 1% lidocaine without vasoconstrictor on each side, using the bilateral pudendal nerve block technique described previously[17]. The patient reported improvement 15 minutes after the procedure. Concomitantly, the dose of pregabalin was increased to 75 mg in the morning and 150 mg at night, fluoxetine was replaced with paroxetine 20 mg, and the patient was referred for mental health assessment in the Psychiatry Department and to Neurology Department for further evaluation of her neurological condition.

The patient returned after 2 weeks for follow-up, complaining of adverse gastrointestinal effects due to pregabalin. She reported an improvement in symptoms after the pudendal nerve block, with an approximate 80% reduction in the frequency of orgasms, *i.e.*, around 3 orgasms/day. In the return visit, the patient expressed intense fear of the recurrence of the symptoms. Again, transcutaneous blockage guided by digital vaginal palpation was performed, as described above, unilaterally on the right side with 1% lidocaine without vasoconstrictor. Pregabalin was administered. During the same visit, the patient stated that she had not engaged in sexual intercourse since the first transcutaneous blockage was performed, due to the lack of sexual desire.

In the third follow-up after the second pudendal nerve blockage on March 27, 2023, the patient reported having performed sexual intercourse with normal sexual response, exhibited sexual desire and arousal, and achieved orgasm. She was then referred to psychological monitoring and physiotherapy. The patient had a consultation session with a psychiatrist on March 31, 2023, when she was switched from fluoxetine 60 mg/day to paroxetine 15 mg/day.

The evaluation by the neurology team was carried out on April 6, 2023, when the patient presented with "pulling/pressure-type" pain that originated from the lumbar region to the buttocks, bilaterally, without irradiation to the legs, with an intensity of 7/10, and with pain in the coccygeal region with 10/10 intensity that worsened with movement. Sensitivity examination showed tactile hypoesthesia in the territories of L5-L4-L3 and S1, with some sites of allodynia. The muscle strength assessment revealed strength grade 4 upon bilateral hip flexion + hip extension + hip abduction and adduction; strength grade 5 in the distal portion of the leg upon bilateral plantar and dorsal flexion, inversion, and eversion; and torque grade 2 upon bilateral abduction and adduction in the two lower limbs. An MRI scan of the lumbosacral spine and bilateral hip, as well as electroneuromyography, was requested. The dose of pregabalin was adjusted to 150 mg per 12 h while the doses of other medications were maintained. Neurosurgical evaluation pointed to the necessity of pudendal block, which should be conducted depending on the PGAD condition.

Upon returning to the AESH on May 8, 2023, she reported excellent adaptation to the use of paroxetine 15 mg, with the only adverse effect being drowsiness. The patient also reported having orgasms only when seated or when the coccygeal region was stimulated. According to her, during penetrative sexual intercourse, she noticed a slight reduction in sensitivity, as well as a decrease in the number of orgasms; however, she was satisfied with the result and was not bothered by this change in the response pattern during intercourse with her partner. As for autoerotism, after sympathetic block, she described difficulty in locating the area of greatest pleasurable sensitivity in the genitalia and the need for more stimuli to reach orgasm. Since the patient no longer reported distress nor complained of intrusive arousal and orgasm, we decided to maintain the prescribed medication and instructed the patient to return for a safety follow-up visit at the AESH together with the mental health and neurology teams.

3. Discussion

This case report sought to demonstrate the clinical management of PGAD in a woman. It is challenging to define the appropriate strategy to control this condition since the laboratory and imaging test results do not always directly lead to the diagnosis[9], complicating the identification of an appropriate treatment regimen. However, it is known that peripheral neurological processes such as congenital anomalies or pudendal nerve injuries can result in the spontaneous activation of sympathetic C sensory fibers related to sexual arousal[18], which could, in part, explain the patient's condition.

After the first pudendal nerve blockage with 1% lidocaine, the patient experienced an immediate reduction in 80% of the referred symptoms, convincing evidence that the same treatment methodology already described in another case report could lead to a rapid improvement of the condition[19]. It is worth mentioning that the patient had undergone an unsuccessful medullary blockage and was also using several pain medications, including centrally-acting neuromodulators. Since she had pelvic pain due to radicular compression radiating to the perineal region, we decided to perform pudendal nerve blockage since PGAD may be associated with pudendal neuralgia[20]. This technique has been reported in a case of PGAD and proved to be effective for the immediate improvement of symptoms[19].

Since the etiology of PGAD is not yet well defined, there is no established protocol for the management of this condition. However, several interventions have been

described in the literature, including discontinuation or prescription of SSRIs, psychoactive drugs, surgical treatment, transcutaneous electrical nerve stimulation, botulinum toxin, physiotherapy, cognitive behavioral therapy, mindfulness techniques, relaxation exercises, among others[2,21-23] (Table 1). The wide range of interventions implies the complexity of this condition and the need for empirical management to control symptom.

Identifying PGAD presents a challenge given its complexity, prompting clinicians to rely on a thorough medical history, physical assessments, and various tests including MRI and neuroelectrophysiology for making diagnosis[24]. These tests serve to differentiate other potential causes, aiding in the diagnostic process of PGAD. A potential direction for exploring the diagnostic techniques for PGAD is integrating neuroelectrophysiology as part of the armada of tests. A comprehensive investigation into the neurological base is essential for the effective management of PGAD. Emphasizing the necessity of a thorough exploration rather than fixating on a single factor will significantly contribute a more in-depth and comprehensive viewpoint for understanding and a therapeutic direction for this condition. This broader investigative approach extends the boundaries of inquiry and enhances the capacity for a nuanced comprehension, enabling more precise and targeted interventions. Ultimately, this approach aims to develop more effective and tailored treatments for individuals affected by PGAD.

Table 1. Factors associated to persistent genital arousal disorder and possible treatments available

Associated conditions
Psychosocial symptom distress
Anxiety symptoms/panic
Depressive symptoms/suicidality
Obsessive-compulsive symptoms
Sexual/emotional/other trauma
Catastrophization/hypervigilance
Relationship adjustment
Overactive/hypertonic pelvic floor muscle dysfunction
Lumbar disc disease Annular tear
Pudendal neuropathy
Sacral Tarlov cyst
Radiculopathy of sacral spinal nerve roots within the cauda equina or sacrum
Use of medications such as trazodone
Use of or discontinuation of SSRIs/SNRIs
Abdominal wall nerve neuroma
Other medical comorbidities
Possible treatments
Psychotherapy and other psychological strategies (e.g., cognitive behavior therapy, decatastrophization, mindfulness, breathwork, self-compassion, etc.)
Sacral/pudendal neuromodulation at the pelvic floor
Dose adjustment of SSRI/SNRIs
Pudendal nerve blockage
Electroconvulsive therapy
Tarlov cyst surgery or aspiration (with or without fibrin glue)
Lumbar disc disease surgery (*e.g.*, laminectomy, discectomy, annuloplasty)

Abbreviations: SSRIs: Selective serotonin reuptake inhibitors; SNRIs: Selective serotonin-norepinephrine reuptake inhibitors. Modified from Goldstein *et al.*[21]

4. Conclusion

The current study demonstrated that a pudendal nerve blockage resulted in an immediate decrease in the related symptoms of PGAD in a woman diagnosed with lumbar disc disease and Tarlov cyst. This clinical case may contribute to the management of neurological conditions that affect the sacral region associated with PGAD and also highlight the significance of the multidisciplinary team involved in the treatment process.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

This case report was approved by the Research Ethics Committee of the FMRPUSP Clinics Hospital (approval ID: CAAE-69898023.0.0000.5440). The participant signed the consent form before participation.

Consent for publication

The participant has given her consent for releasing her data.

Availability of data

Not applicable.

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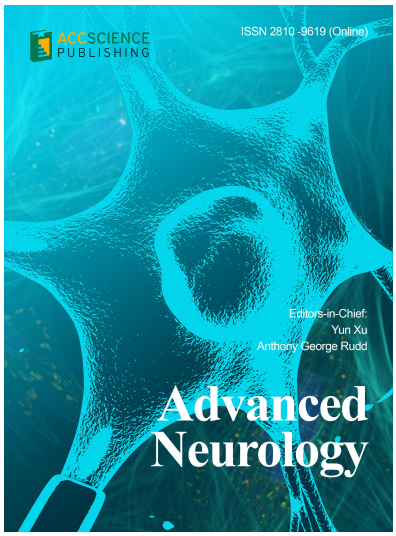

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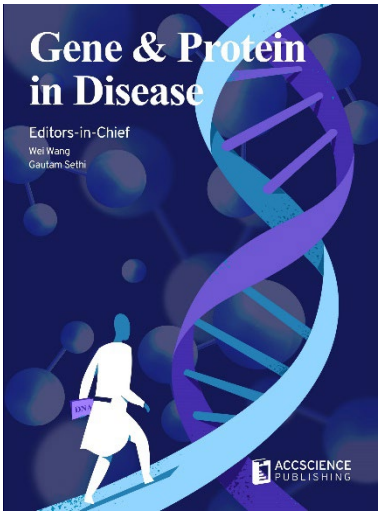

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