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## Radiation-induced cardiovascular disease: an overlooked role for DNA methylation?

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### ABSTRACT

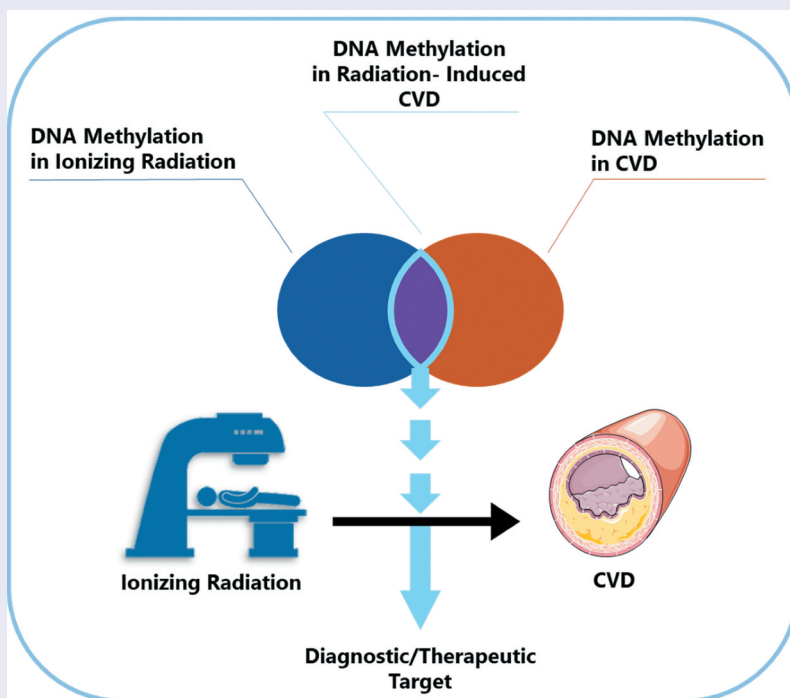
Radiotherapy in cancer treatment involves the use of ionizing radiation for cancer cell killing. Although radiotherapy has shown significant improvements on cancer recurrence and mortality, several radiation-induced adverse effects have been documented. Of these adverse effects, radiation-induced cardiovascular disease (CVD) is particularly prominent among patients receiving mediastinal radiotherapy, such as breast cancer and Hodgkin's lymphoma patients. A number of mechanisms of radiation-induced CVD pathogenesis have been proposed such as endothelial inflammatory activation, premature endothelial senescence, increased ROS and mitochondrial dysfunction. However, current research seems to point to a so-far unexamined and potentially novel involvement of epigenetics in radiation-induced CVD pathogenesis. Firstly, epigenetic mechanisms have been implicated in CVD pathophysiology. In addition, several studies have shown that ionizing radiation can cause epigenetic modifications, especially DNA methylation alterations. As a result, this review aims to provide a summary of the current literature linking DNA methylation to radiation-induced CVD and thereby explore DNA methylation as a possible contributor to radiation-induced CVD pathogenesis.



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## Introduction

Mediastinal radiotherapy is an effective means employed in the treatment of breast cancer, lung cancer, oesophageal cancer, thymoma, and mediastinal lymphoma (Hodgkin's lymphoma (HL), primary mediastinal B-cell lymphoma (PMBCL), and T-lymphoblastic lymphoma) [1–5]. However, radiotherapy comes with definite side effects such as radiation-induced cardiovascular disease (CVD). In breast cancer, significantly higher rate of major coronary events were observed in irradiated left-sided breast cancer patients over their right-sided breast cancer counterparts [6]. A reason for this difference could be the different mean heart doses after left sided and right sided breast cancer radiotherapy being 3.6 and 1.9 Gy respectively [6–8]. As for Hodgkin's lymphoma, treatment most commonly employs, in addition to radiotherapy, cardiotoxic chemotherapeutic agents e.g. anthracycline. Consequently, lymphoma survivors have a 5.3–7.3 times increased risk of death from cardiac mortality when compared to the general population [9,10]. The mechanisms underlying radiation-induced CVD are not completely understood. Moreover, a possible contribution of epigenetics mechanisms has recently been proposed.

Previous research has linked altered DNA methylation to the development of many diseases including CVD [11,12]. Conversely, ionizing radiation has been shown to cause DNA methylation alterations both *in vitro* and *in vivo* [13–16]. Considering the common occurrence of DNA methylation alterations in CVD and after ionizing radiation exposure, we suggest a previously unaddressed involvement of DNA methylation in radiation-induced CVD.

## Radiation in cancer therapy

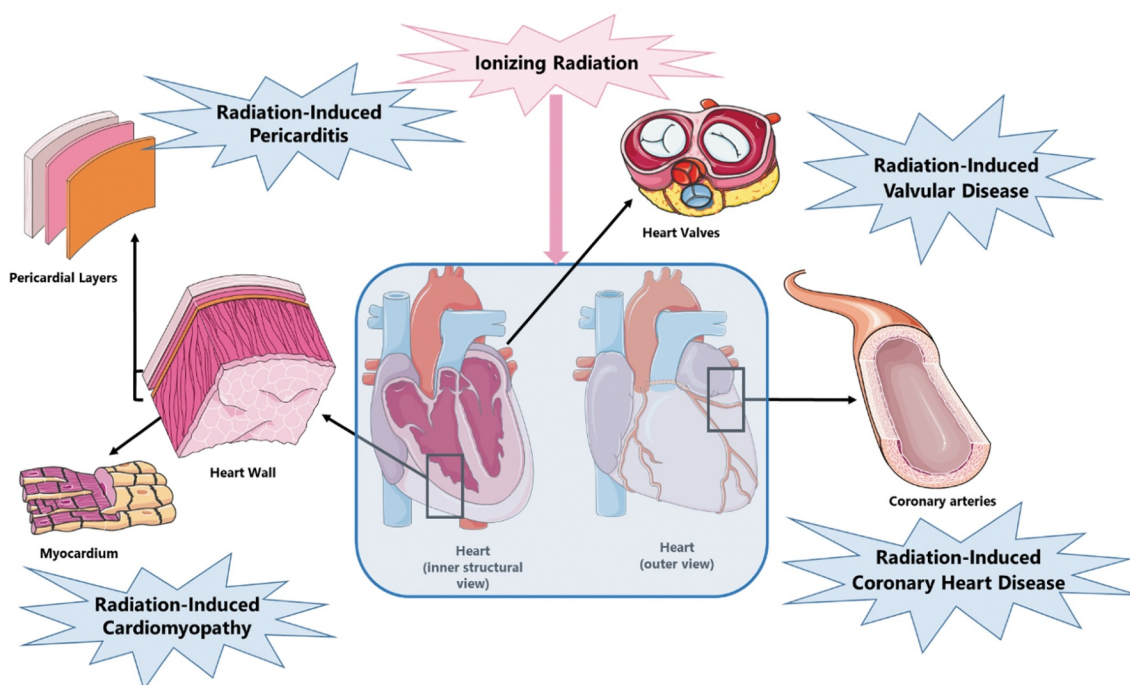
Radiation therapy employs high doses of ionizing radiation to kill cancer cells and shrink tumours. Radiation exerts its signature lethal cellular effects either directly or indirectly. Indirect effects involve the hydrolysis of water molecules to produce free radicals which can damage cellular compounds, such as DNA [17]. Several factors affect the biological effectiveness of radiation, namely the linear

energy transfer (LET), total dose, fractionation rate, and radiosensitivity of the targeted cells or tissues [18]. LET is the energy transferred to the tissue by ionizing radiation (IR) per unit tract length. Simply, more charged, slower moving alpha particles and neutrons have higher LET and subsequently deliver more cell damaging energy to tissues than lesser charged, faster moving X-rays and  $\gamma$ -rays [19].

Despite the beneficial outcomes of cancer radiotherapy, cardiac toxicity occurring due to incidental heart irradiation is an undesirable healthy tissue side effect [7]. Previously, the heart was considered insensitive to radiation doses less than 30 Gy in accordance to the law of Bergonié and Tribondeau [20,21]. According to which, the degree of radiosensitivity of a biological tissue is dependent on its growth rate and degree of differentiation. Consequently, the heart with its non-dividing tissue was dubbed rather non-radiosensitive [21]. However, it was shown that the incidence of excess major coronary events increased by 7.4% per 1 Gy increase in the mean heart dose delivered by breast cancer radiotherapy with no observed safe threshold of dose [6].

## Radiation-induced CVD manifestations

Radiation-induced CVD can manifest in different ways: coronary heart disease, pericarditis, cardiomyopathy and valvular heart disease (Figure 1). Due to contemporary radiation-sparing techniques including prone position radiotherapy, image- and dose- guided radiotherapy, intensity modulated radiation therapy (IMRT), stereotactic body radiation therapy (SBRT) and deep inspirational breath hold (DIBH), the incidence of acute pericarditis and cardiomyopathy has been reduced [22–25]. Consequently, coronary heart disease accounts for most of the cardiac mortality attributable to radiotherapy with the first signs of cardiac toxicity typically appearing after 10–15 years of follow-up [26–28]. Risk factors for the development of radiation-induced CVD strongly overlap with conventional risk factors for CVD, such as diabetes, hypertension, obesity, smoking, chronic obstructive pulmonary disease (COPD) and hypercholesterolemia. Additionally, therapy-dependent factors such as the cumulative dose from radiotherapy,



**Figure 1.** Radiation-induced CVD presentation.

Made with Microsoft Powerpoint using images from Server Medical Art (<https://smart.servier.com/>).

chemotherapeutics (e.g. anthracyclins) and regular analgesic use represent additional risk factors [6,29].

### **Radiation-induced coronary heart disease**

Radiation-induced coronary heart disease (CHD) - also referred to as myocardial ischaemia- is caused by atherosclerosis of medium and large vessels. The pathogenesis of atherosclerosis involves a complex interplay of lipid accumulation, local inflammation and smooth muscle cell proliferation resulting in the formation of atherosclerotic plaques. Stable atherosclerotic plaques may narrow the lumen and hamper blood flow. However, unstable plaques may rupture and cause total occlusion of blood flow through thrombosis, leading to myocardial infarction. The risk of developing radiation-induced CHD is proportional to dose. This risk also increases within the first 5 years after exposure to radiation and continues for at least 20 years. In addition, radiation-induced CHD occurs at  $\leq 10\%$  of the tolerance dose of other cardiac tissues (e.g. 36–40 Gy mean heart dose for the pericardium and 40 Gy for the myocardium) [6,30,31].

Interestingly, similar radiation-induced atherosclerotic changes in the carotid artery were observed in Hodgkin's lymphoma patients who received neck radiotherapy [32,33]. This arterial thickening leads to an increased relative risk for transient ischaemic attack or ischaemic stroke ( $\geq 2$  general population risk) [34,35].

### **Radiation-induced pericarditis**

Radiation-induced pericarditis describes a state of acute or delayed onset pericardial inflammation. Acute radiation-induced pericarditis is an early side effect to high radiation doses. The tolerance dose of human pericardium is estimated to be a mean heart dose higher than 36 or 40 Gy or a dose of 50 Gy administered to more than 30% of the heart. Consequently, due to current radiation-sparing techniques, acute radiation-induced pericarditis is considered very rare. On the other hand, chronic radiation-induced pericarditis is one of the most common manifestations of radiation-induced CVD presenting months to years after radiotherapy. However, its risk has also decreased significantly due to the same radiation-sparing

techniques. Chronic radiation-induced pericarditis patients develop fibrous thickening of the pericardium even before manifestation of symptoms. This pericardial thickening might progress to chronic constrictive pericarditis which could then lead to intractable heart failure and will require pericardiectomy when it becomes symptomatic [30,36,37].

### **Radiation-induced cardiomyopathy**

Radiation-induced cardiomyopathy most commonly occurs after previous valvular disease or myocardial infarction. The tolerance dose of the human myocardium is approximately 40 Gy. Consequently, pronounced radiation-induced cardiomyopathy occurs at higher doses. The most probable mechanism behind radiation-induced cardiomyopathy is radiation-induced microvasculature damage leading to capillary loss and myocardial hypoxia and cell death. Dead cells are replaced by fibrotic tissue leading to a reduction of myocardial elasticity and distensibility [30,31].

### **Radiation-induced valvular disease**

Radiation-induced valvular disease risk is dose dependent with delayed onset (up to 20 years after radiotherapy). It leads to endocardial fibrosis which starts by thickening and calcification of the valvular endocardium [38,39].

### **Mechanisms of radiation-induced CVD**

Radiation-induced CVD pathophysiology has been extensively described in literature [30,31,40–45]. Inflammatory changes as well as reactive oxygen species (ROS) production appear to be the main causes of the early radiation-induced cardiac tissue damage. On the other hand, persistence of this state of inflammation and oxidative stress results in the delayed radiation-induced tissue damage [44–47].

An early event in radiation-induced cardiac effects is NF- $\kappa$ B activation. NF- $\kappa$ B is a transcription factor implicated in the regulation of immune cell maturation, cell survival, and inflammation signalling pathways [48]. IR activates NF- $\kappa$ B through ROS and double stranded

breaks (DSBs) as well as damage-associated molecular patterns (DAMPs) released from stressed or dying cells [42]. DAMP binding to endothelial cells also activates MAPK, and interferon regulatory factor 3 (IRF3) signalling [42,49]. This leads to the expression of various pro-inflammatory cytokines (e.g. Interleukins 1 & 6, interferon- $\gamma$  (IFN- $\gamma$ ), and Tumour Necrosis Factor (TNF- $\alpha$ )), chemokines (e.g. Monocyte chemoattractant protein-1 or MCP-1), cell adhesion molecules (e.g. Vascular cell adhesion molecule 1 or VCAM-1 and E-Selectin) and matrix metalloproteinases. This ultimately initiates an acute inflammatory state with endothelial activation and inflammatory cell recruitment within minutes of IR exposure. The recruited inflammatory cells (mainly neutrophils) add to the inflammatory state and secrete pro-fibrotic transforming growth factor beta (TGF- $\beta$ ). TGF- $\beta$  has been shown to initiate myocardial remodelling by inducing cardiac fibroblast differentiation into myofibroblast with increased collagen deposition and eventual fibrosis [50].

In addition to chronic inflammation, chronic oxidative stress is an important pathophysiologic mechanism of radiation-induced CVD. As previously mentioned, IR-induced ROS play a role in the activation of NF- $\kappa$ B. In addition, ROS upregulate several enzymes such as nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), lipoxygenases (LOXs), nitric oxide synthase (NOS), and cyclooxygenases (COXs). These enzymes contribute to the acute and chronic effects of oxidative stress by inducing production of inflammatory and pro-fibrotic cytokines, prostaglandin production, lipid peroxidation and inhibition of DNA repair [51].

Interestingly, recent studies showed an activation of nucleotide-binding domain and leucine-rich-repeat-containing family pyrin 3 (NLRP3) inflammasome after thoracic irradiation of C57BL/6 mice [52]. Irradiation of THP-1 monocytes led to a similar inflammasome activation resulting in increased expression of interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 [52,53]. This is especially important as IL-1 secretion was suggested to participate in the development of radiation-induced CVD. This was further evidenced by amelioration of radiation induced sustained expression of inflammatory mediators in mice after

administration of IL-1 $\beta$  blocker directly after irradiation for 2 weeks [54,55].

This state of inflammation and oxidative stress is suggested to initiate a number of pathophysiologic dysfunctions. The first is cell death and premature endothelial senescence [56]. Aside from the direct apoptotic effects of radiation, irradiation of cardiac myocytes stimulates calcium release from the endoplasmic reticulum. This causes mitochondrial calcium overload, mitochondrial membrane swelling and release of apoptotic factors [44]. On the other hand, endothelial cell senescence occurs as a result of activation of Ataxia telangiectasia mutated (ATM)/p53/p21 and protein Kinase B/phosphatidylinositol 3-kinase/mechanistic target of rapamycin (Akt/PI3K/mTOR) pathways [42,56]. In addition, IL-6 induced by NF- $\kappa$ B activation has been previously identified as a critical controller of autocrine senescence [57–59]. Finally, accelerated telomere shortening induced by oxidative stress and mitochondrial dysfunction also contribute to accelerated endothelial senescence [57–60].

The second contributing pathophysiologic dysfunction is impaired endothelium-dependent relaxation of blood vessels. This is mediated mainly by endothelial nitric oxide synthase (eNOS) uncoupling and direct inactivation of NO by superoxide radicals leading to diminished vasodilation [61–64]. Senescent endothelial cells also contribute to decreased NO production due to their lowered eNOS activity [65]. In addition, decreased vasodilating prostacyclin and increased vasoconstrictive endothelin-1 and angiotensin-II levels contribute to chronic vasoconstriction [66,67]. The third contributing pathophysiologic dysfunction is mitochondrial dysfunction. Mitochondrial DNA is especially sensitive to radiation-induced damage due to its limited repair capacity, lack of protective histones, a high exon/intron ratio and its close proximity to the electron transport chain [42]. Furthermore, radiation-induced ROS can cause mutations in mitochondrial DNA as well as damage or alter the expression of proteins required for critical mitochondrial and cellular functions. The damaged mitochondria then generate more ROS which starts a vicious cycle of mitochondrial ROS generation [51,68].

The final contributing pathophysiologic dysfunction is initiation of a pro-coagulative and pro-

thrombotic state. Endothelial cell damage with secreted pro-inflammatory cytokines induces the secretion of von Willebrand factor (vWF), platelet-activating factor and tissue factor while reducing thrombomodulin and prostacyclin production [42]. In addition, TGF- $\beta$  released from irradiated endothelial cells induces fibrogenic activation of vascular smooth muscle cells leading to increased proliferation and migration [69]. This, along with decreased NO availability, leads to increased platelet aggregation and thrombus formation [42]. Recently, the European Society for Medical Oncology (ESMO) proposed aspirin as an antiplatelet as one of the cardioprotective treatments for patients receiving radiotherapy and having a high risk for CVD [70]. However, up to this moment, antiplatelet therapy has not proven to be beneficial in radiation-induced CVD [71–73]. Consequently, non-traditional therapies against CVD may need to be further investigated.

### **DNA methylation: a possible overlooked mechanism in radiation-induced CVD?**

Epigenetic modifications involve different mechanisms, such as histone modifications, changes in DNA methylation and regulation of gene expression through micro-RNAs. Epigenetic modifications induce changes in gene expression that can occur without any changes in the primary DNA sequence. Moreover, epigenetic alterations could be transmitted to future progeny [74]. Alterations in DNA methylation are the most widely researched epigenetic changes and will be the focus of the current review.

#### **DNA methylation**

DNA methylation involves methylation of a cytosine base in a CpG dinucleotide to produce 5-methyl cytosine (5-mC). A process that is essential for silencing of transposable elements, genomic imprinting and X-chromosome inactivation [75]. 5-mC represents only ~1% of nucleic acids in the genome [76–78]. This low CpG frequency occurs due to cytosine deamination. Spontaneous cytosine deamination yields uracil. This uracil is then repaired by uracil glycosylase to cytosine. On the other hand,

deamination of 5-mC yields thymine which is not corrected by uracil glycosylase leading to a C/T transition. This C/T transition is the most frequent mutation observed in human diseases occurring 10–50 times more frequently than all other base transitions. Consequently, cytosine deamination is the main reason for the observed frequency of CpG dinucleotides being lower than the expected CpG frequency in the absence of deamination [79,80]. The biological significance of this C/T transition is especially apparent when considering arginine deamination. The codon for arginine amino acid (CGA) can be changed upon oxidative deamination of 5-mC into a stop codon (TGA). The premature appearance of a stop codon leads to the production of truncated and usually inactive proteins [81].

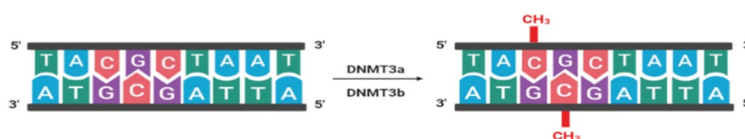
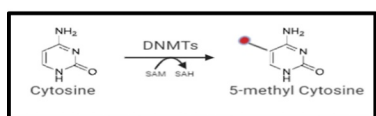
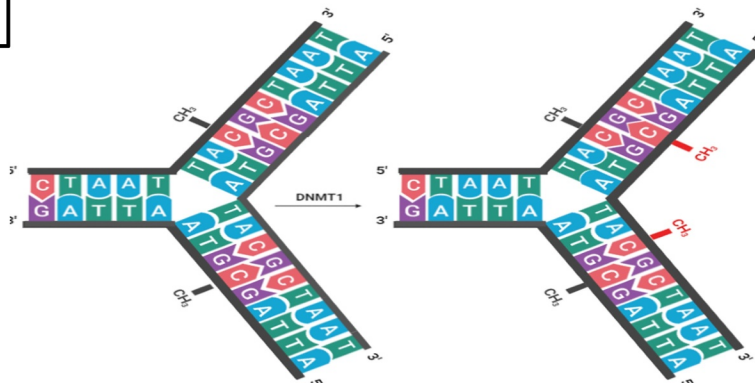
The majority of CpG dinucleotides in the human genome are heavily methylated (~70%) with the rest being unmethylated and mainly part of so called CpG islands (CGIs). CGIs are defined as regions of DNA (>200 bps) that have a GC content of at least 50%, observed CpG frequency of at least 60% of the expected CpG frequency, lack methylation and consequently are not transcriptionally silenced [82]. The expected CpG frequency in the previous definition is the CpG frequency if no cytosine deamination occurs. This high content of CpGs allows for DNA methylation-mediated regulation of gene expression. About 70% of gene promoters are associated with these CGIs and around 50% of these CGIs contain transcription start sites [83]. Hyper- or hypomethylation of DNA has long been considered to inhibit or activate gene expression, respectively. However, the relationship between the DNA methylation state and gene expression is actually more complex as will be discussed later. Nonpromoter CGIs are CGIs that can be found in inter- and intragenic sequences. These CGIs represent either alternative transcription start sites of protein-coding genes or noncoding RNAs [84,85]. Intragenic or gene body CGIs that are present in actively expressed genes show increased DNA methylation. This can be due to gene body CGI's ability to block transcription at intragenic promoters, affect intragenic repetitive element

activity and alter mRNA splicing by destabilizing nucleosomes at intron-exon junctions [86]. In general, methylation at CGIs is relatively stable in normal healthy tissue with major methylation pattern alterations associated with various disease states including cancer [87].

Historically, DNA methylation can be classified into two main types: *De novo methylation* which happens mainly in the developing embryo and *maintenance methylation* which maintains the methylation patterns from the parent strand in the daughter strand during replication. Consequently, DNA methylation is an epigenetic pattern that is maintained in cellular progeny and is normally stable in non-dividing cells [88,89].

DNA methylation is carried out by the action of DNA methyltransferases or DNMTs. Initiation of DNA methylation in the growing embryo during implantation occurs under the effects of the *de novo* DNMTs (DNMT-3a and -3b) with the help of DNMT3 like (DNMT3l). Here, the *de novo* DNMTs transfer methyl groups from S-adenyl methionine (SAM) to cytosines of the unmethylated DNA strand. DNMT3l is a DNMT3 family member which is incapable of individual methyltransferase activity but increases the activity of DNMT-3a and -3b by up to threefold [89]. Following this initial methylation step, the maintenance DNMT (DNMT1) then replicates the methylation pattern in following cell divisions. There, DNMT1 methylates hemimethylated CpG dinucleotides at the replication fork where newly biosynthesized DNA strands are directly methylated (Figure 2) [78,89,90]. This 'maintenance' thereby allows inheritance of the methylation patterns of the parent cell. The addition of a methyl group to C5 of a cytosine leads to the production of a stable covalent bond that requires a high degree of energy to be broken. An observation that led to the mistaken belief of the irreversibility of DNA methylation [91,92].

While DNA methylation patterns are fairly stable over time, DNA can be demethylated as well. DNA demethylation can occur through passive and active mechanisms. Passive demethylation occurs by inhibition of -or reduction in- DNMT1 levels. This inhibition or reduction of DNMT1 allows newly incorporated cytosine to remain

**a) De Novo Methylation****b) Maintenance Methylation**

**Figure 2.** The writers of DNA methylation. DNMTs transfer a methyl group from SAM to the 5<sup>th</sup> carbon of cytosine. (A) *De novo* methylation process in the developing embryo by the action of DNMT3a and DNMT3b. (B) Maintenance methylation process during DNA replication to maintain the methylation profile in the daughter cells by the action of DNMT1 present at the replication fork on hemimethylated DNA. DNMT: DNA methyltransferase, SAM: S – adenosyl methionine, SAH: S-adenosyl-L-homocysteine. Made with Biorender (biorender.com).

unmethylated thereby causing a replication-dependent dilution of 5-mC [78,93]. Active demethylation involves the modification of the methyl group of 5-mC by enzymatic action and subsequent restoration of the non-methylated cytosine by DNA repair. Active DNA demethylation is mainly mediated by the actions of ten-eleven translocation (TET) enzyme [83].

### **Does radiation impact DNA methylation and how?**

Radiation has been shown in previous studies to cause DNA methylation alterations [15,94–98]. However, the interplay between IR and DNA methylation is highly complex and may be tissue-dependent as well as model and strain-specific [99]. A summary of the literature [13–16,94–96, 100–109] addressing the effect of radiation on DNA methylation is provided in Table 1 of Supplementary Materials.

Research addressing radiation-induced DNA methylation alterations was found to involve different radiation types, doses and sampling times. Subsequently, the task of arriving to a simple conclusion regarding the exact effects of IR on DNA methylation becomes extremely difficult. Some

studies address the effects of IR exposure during spaceflight. Subsequently, those employ low doses of high LET protons and heavier high atomic number and energy (HZE) ions such as <sup>56</sup>Fe radiation [94,96,107]. On the other hand, studies that address the effects of the medicals application of radiation tend to use different types and doses of radiation in an attempt to mimic the doses regularly received by patients. In addition, even among those studies, different doses, dose intensities and study durations are employed. This is because of the varying aims of these studies which range from the investigation of radiation-induced carcinogenesis, radiation-induced genomic instability and bystander effects [13–15,95,101,102,109,110] to cellular responses to radiation and radiosensitivity [100,103,104,108]. In addition, some studies addressed the difference between the effects of IR on somatic and germinal tissues [16].

Another important and somewhat confounding aspect of the effects of IR on DNA methylation is the common use of *in vivo* animal models with rather short lifespan compared to humans. This is because the late effects of IR tend to develop in humans several years after the initial radiation. Consequently, investigating these late side effects in animal models requires taking into



**Table 1.** Functional analysis of CVD differentially methylated genes in literature.

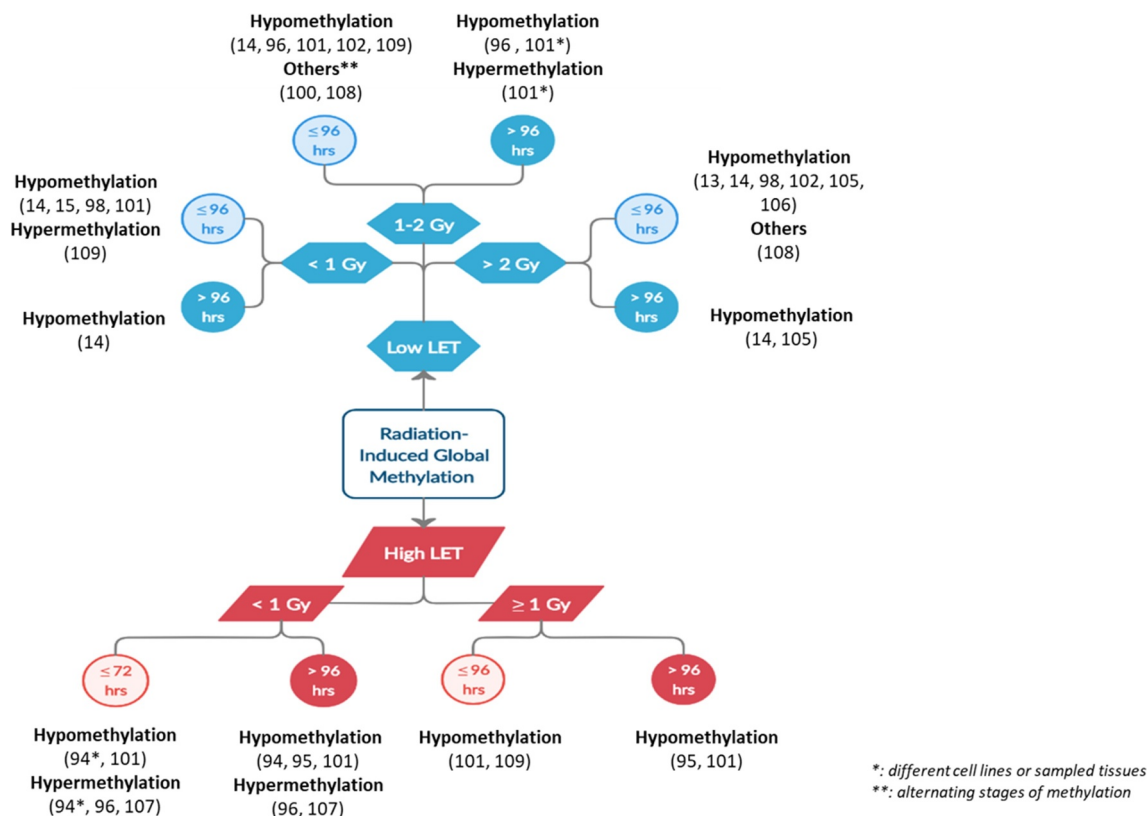
PANTHER Pathways	Number of genes	raw P-value	False Discovery Rate (FDR)
JAK/STAT signalling pathway	3	0.0000	0.0010
Interferon-gamma signalling pathway	3	0.0001	0.0035
PI3 kinase pathway	3	0.0003	0.0112
Interleukin signalling pathway	3	0.0012	0.0393
PDGF signalling pathway	3	0.0048	0.1300
Angiogenesis	3	0.0076	0.1770
TGF-beta signalling pathway	2	0.0222	0.4040
Inflammation mediated by chemokine and cytokine signalling pathway	3	0.0214	0.4380
Androgen/estrogene/progesterone biosynthesis	1	0.0295	0.4830
5-Hydroxytryptamine degradation	1	0.0494	0.7360
Adrenaline and noradrenaline biosynthesis	1	0.0667	0.7810
Axon guidance mediated by Slit/Robo	1	0.0624	0.7870
CCKR signalling map	2	0.0606	0.8280
Dopamine receptor mediated signalling pathway	1	0.1250	1.0000
p53 pathway	1	0.1840	1.0000
Wnt signalling pathway	2	0.1660	1.0000
VEGF signalling pathway	1	0.1470	1.0000
p53 pathway feedback loops 2	1	0.1110	1.0000
Ras Pathway	1	0.1590	1.0000
Oxidative stress response	1	0.1210	1.0000
Gonadotropin-releasing hormone receptor pathway	1	0.4170	1.0000
Endothelin signalling pathway	1	0.1740	1.0000
EGF receptor signalling pathway	1	0.2710	1.0000
Cadherin signalling pathway	1	0.3100	1.0000
Blood coagulation	1	0.1030	1.0000
Apoptosis signalling pathway	1	0.2350	1.0000
Alzheimer disease-presenilin pathway	1	0.2500	1.0000

consideration the equivalent age of the animals [111]. This is indeed difficult as there are wide variations in the developmental durations and phases of these animals versus humans. Consequently, special considerations to the exact developmental stage of the used animal model are necessary to correlate the results to human stage [112].

Despite these difficulties, these studies share trends in DNA methylation alterations in response to IR analysed on both a global and a gene-specific scale. Global DNA methylation offers a representation of the total 5-mC content in the genome, but lacks gene-specific information. On the other hand, gene-specific methylation focuses on the methylation alterations of specific genes. In Figure 3, we attempt to summarize the studies addressing the effect of

radiation on DNA methylation from a global DNA methylation perspective. In Figure 3, we observe a general trend of radiation-induced global hypomethylation which persists for several months in a number of studies employing animal models. This suggests a link between DNA methylation alterations and IR-induced late effects observed years after radiotherapy [14,94,96,110]. Global hypomethylation is mainly evaluated by the methylation of DNA repetitive elements (REs) which account for about 50% of the human genome. Of which, Alu element (Alu) and long interspersed element-1 (LINE-1) are the most abundant human RE sequences. Hypomethylation of these REs will therefore lead to their reactivation, retrotransposition and resultant genomic instability [113]. On the other hand, attempting a similar overview for gene-specific methylation is more difficult. Different studies reported different sets of methylated genes with no clear patterns. Of note, IR was reported to elicit both hyper- and hypomethylation of individual genes. However, overall radiation-induced gene-specific hypermethylation occurred at a higher frequency than gene-specific hypomethylation.

Within these literature studies, a number of possible mechanisms have been put forward (Figure 4). The first mechanism involves ROS produced by radiation-induced water hydrolysis. These ROS are able to alter DNA methylation patterns through several ways [114]. ROS may act directly on the DNA by oxidizing the guanine base in CGIs forming 8-oxo-2'-deoxyguanosine (8-OHdG), which can be repaired by its removal by 8-oxoguanine DNA glycosylase (OGG1) followed by base excision repair (BER). If not repaired, 8-OHdG can prevent the methylation of the adjacent cytosine leading to subsequent hypomethylation. Alternatively, ROS may directly convert 5-mC to 5-hydroxyl mC by interaction of DNA with hydroxyl radicals. ROS may affect DNA methylation indirectly by altering the activity of methylation related enzymes [114]. However, ROS effects on DNMTs seem to be bidirectional. ROS can lead to hypermethylation by upregulating DNMTs or increasing their recruitment by H<sub>2</sub>O<sub>2</sub>. ROS can also lead to hypomethylation by reducing the availability of SAM



**Figure 3.** Overview of the shared outcomes of the different studies involving radiation-induced global methylation. Made with creately (creately.com) and Microsoft Powerpoint.

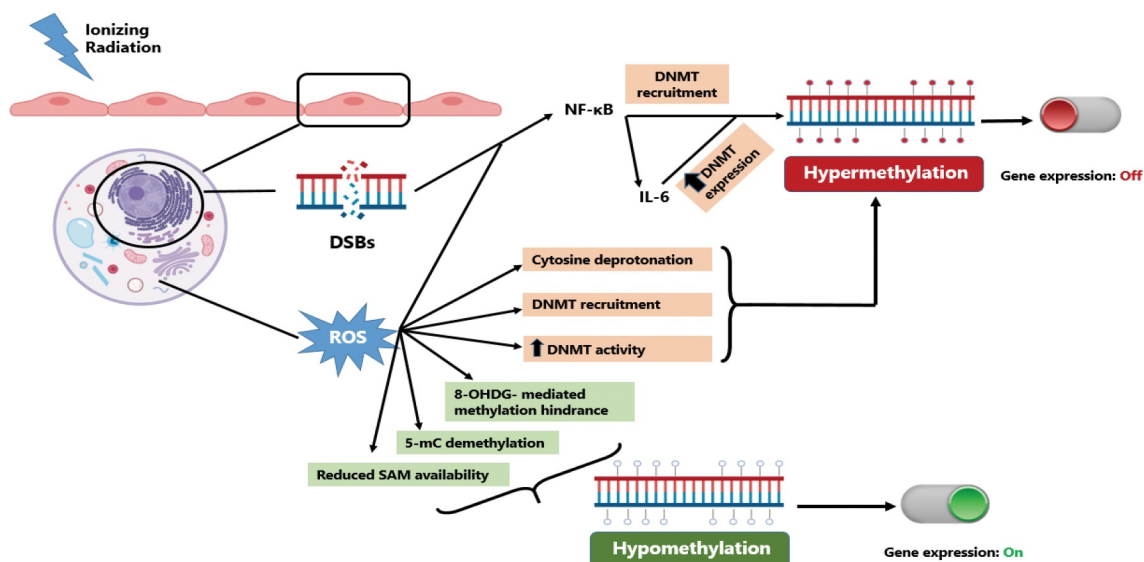
which is an essential cofactor for DNMT activity. Finally, other studies have shown that ROS can lead to DNA hypermethylation due to their nucleophilic action on the 5 position of cytosine molecule leading to its deprotonation and accelerating its reaction with SAM [115,116]. In summary, ROS can lead to hypomethylation by 8-OHdG formation, 5-mC hydroxylation or reducing SAM availability. On the other hand, ROS can also lead to hypermethylation by DNMT upregulation or increased recruitment as well as 5-mC deprotonation. Despite the counter intuitiveness of ROS having opposing effects on DNA methylation, this ‘dual’ effect may contribute to the observed similar opposing effects of radiation on DNA methylation.

Secondly, radiation can also cause DNA hypermethylation by activation of NF- $\kappa$ B [117--117–120]. NF- $\kappa$ B activation leads to alterations in DNA methylation through two different mechanisms. Firstly, the RelA/p65 subunit of NF- $\kappa$ B can directly recruit DNMT-1 to

chromatin [121]. Secondly, NF- $\kappa$ B regulates DNA methylation indirectly by the production of Interleukin-6 (IL-6), which has been shown to also regulate DNMT1 expression leading to increased DNMT1 activity [122–124]. This increased availability/activity of DNMT1 subsequently leads to hypermethylation.

### **DNA methylation and CVD; possible disease collaborator?**

A number of studies have linked DNA methylation alterations to CVD development [125--125–157]. The focus of these studies is mostly atherosclerosis as it is the underlying cause of most CVDs [158–164]. In most cases, global DNA hypomethylation has been observed in atherosclerosis [165–170]. This state of hypomethylation was detectable in atherosclerosis prone murine aorta even before the development of atherosclerosis [171]. In addition, gene specific hypermethylation has also been



**Figure 4.** Possible mechanisms of radiation-induced DNA methylation alterations. incident ionizing radiation leads to the production of DSBs in affected cells. these DSBs as well as radiation-induced ROS can lead to NF-κB activation which in turn can lead to DNA hypermethylation and inhibition of gene expression. on the other hand, radiation-induced ROS can cause varying DNA methylation alterations by multiple mechanisms leading to either hypo- and hyper-methylation with differential regulation of gene expression. DSBs: double strand breaks, ROS: reactive oxygen species, 8-OHDG: 8-oxo-20-deoxyguanosine, 5-mC: 5-methyl cytosine, SAM: S-adenosyl methionine, DNMT: DNA methyltransferase.

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observed in atherosclerosis [147,151,156]. Interestingly, focal gene-specific hypermethylation offers higher biological relevance than that of global hypomethylation [162].

We examined the literature concerning differentially methylated genes in CVD (summarized in Table 2 of Supplementary Materials). In most cases, identified differentially methylated genes are associated with key elements in CVD pathogenesis, such as lipid metabolism, inflammation, oxidative stress, atherosclerosis and endothelial cell dysfunction. This consequently points to a contribution of DNA methylation to CVD pathophysiology. However, care should be taken when translating DNA methylation data from these studies to clinically relevant biomarkers. This is in part due to the fact that the effect of DNA methylation on gene expression is of a complex nature. Several studies have pointed out that the state of CpG methylation does not always predict the state of gene expression. Indeed, examination of the DNA methylation of primary human fibroblasts showed that CpG methylation alterations did not always translate to changes in gene expression [172,173].

Furthermore, it was found that better correlation with gene expression was encountered with certain genomic positions (first intron) or associated with chromatin states, particularly those that are representative of active chromatin and transcribed regions [172,174,175]. Another confounder is that many studies investigating differential methylation in CVD are based on a limited, rather low, number of samples which casts doubt on the capacity to extrapolate the results. Therefore, care must be taken when interpreting the results originating from low sample size or those not backed up with gene expression analyses.

The association between altered DNA methylation and CVD is further proven in experimental models of atherosclerosis. Dunn et al. have shown DNMT inhibitor 5-Aza-2'-deoxycytidine (also known as decitabine) to be effective atheroma preventive measures in a ApoE<sup>-/-</sup> mouse model of atherosclerosis [176]. In addition, decitabine was shown to decrease atherosclerosis development in LDLr<sup>-/-</sup> mice through decreased macrophage inflammation and suppressed macrophage endoplasmic reticulum stress [177]. This could be

explained by decreased methylation and subsequent increased expression of liver X receptor  $\alpha$  (LXR $\alpha$ ) and peroxisome proliferator-activated receptor  $\gamma$ 1 (PPAR $\gamma$ ). LXR $\alpha$  and PPAR $\gamma$  are atheroprotective as they regulate lipid metabolism and macrophage inflammation [178–180]. The interested reader is referred to a number of reviews regarding the use of DNA methylation inhibitors as candidates drugs for treating atherosclerosis [181–183].

### **Functional analysis of differentially methylated genes in CVD**

In an attempt to validate the involvement of DNA methylation in CVD pathogenesis, we performed a functional analysis of genes reported in literature to be differentially methylated in CVD (Table 1). This was done by investigating these genes in the pathway analysis functionality of PANTHER (protein analysis through evolutionary relationships) classification system [184,185]. Our PANTHER analysis of differently methylated genes in Table 1 reveals that these genes were connected to a number of pathophysiological mechanisms of CVD, such as inflammation, oxidative stress and endothelial activation. In addition, four pathways showed statistically significant pathway involvement, namely Janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling pathway, interferon-gamma (INF- $\gamma$ ) signalling pathway, phosphoinositide 3 kinase (PI3K) pathway and interleukin signalling pathway. This should come as no surprise as these pathways are associated with cardiac myocyte response to injury and stress as well as cardiovascular inflammation [186–194]. Activation of JAK/STAT signalling contributes to atherosclerosis development by aiding immune cell recruitment and vascular smooth muscle proliferation, hypertrophy, and migration [195]. In addition, STAT1 synergizes with NF- $\kappa$ B in its inflammatory signalling [196]. Conversely, IFN- $\gamma$  promotes endothelial cell adhesion, immune cell recruitment with a conflicting effect on foam cell formation [197–199]. Also, PI3K catalyzes second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3) production. In the heart, four isoforms (PI3K $\alpha$ , PI3K $\beta$ , PI3K $\delta$ , and

PI3K $\gamma$ ) are differentially expressed in different cell subsets (cardiomyocytes, fibroblasts, endothelial cells and vascular smooth muscle cells). Subsequently, they are associated with varying effects on myocardial contractility, physiological growth and pathobiological remodelling as well as smooth muscle cell and immune cell migration [200–202]. In addition, PI3Ks function as scaffolding proteins in cardiac excitation-contraction coupling and autophagy [201]. Finally, interleukins are a family of cytokines strongly associated with chronic inflammation and atherogenesis. Some interleukins can have proatherogenic effects while fewer interleukins can have atheroprotective effects [197,203–208]. These DNA methylation alterations in pathways associated with atherogenesis suggest a usefulness for DNA methylation based CVD biomarkers. First, Istas et al. found that differentially methylated regions in BRCA1 and CRISP2 genes were reproducibly differentially methylated in independent atherosclerotic human aorta tissue and human carotid plaque samples [156]. In addition, these methylation changes at BRCA1 and CRISP2 genes were consistently associated with subclinical atherosclerosis in an independent sample cohort of middle-aged men. This study provides a concrete example of how gene-specific methylation could be used to monitor the development of atherosclerosis, even before the condition is clinically diagnosable [156]. Second, p16<sup>INK4a</sup> was found to be differentially methylated by IR while also influencing epicardial adipose tissue development and subsequent CVD risk [209]. This intersection of gene-specific methylation alterations observed after radiation and associated with CVD could offer an untapped source of functional biomarkers.

### **Conclusions and future directions**

DNA methylation is an epigenetic mechanism that has been shown to be implicated in the pathogenesis of many diseases. In healthy individuals, the CpG islands in promoter regions are normally non-methylated thereby allowing their associated genes to be transcriptionally active. For the rest of the genome, CpG dinucleotides are normally methylated. Predictably, alterations in DNA methylation lead to changes in gene expression.

Ionizing radiation has been shown in several studies to cause DNA methylation alterations; most commonly global DNA hypomethylation and gene-specific hypermethylation. However, studies investigating radiation-induced DNA methylation alterations vary in radiation LET and dose, type of investigated model as well as the sampling timeline. These variations make it difficult to answer important questions regarding the possible differences in effect on DNA methylation between high and low LET radiation, as well as if there is a dose threshold that needs to be reached to cause DNA methylation alterations. However, the persistence of radiation-induced DNA methylation alterations after IR exposure suggests the involvement of these DNA methylation alterations in radiation-induced late effects.

On the other hand, several studies reported global hypomethylation in CVD and specifically in atherosclerosis. In addition, hyper- and -less commonly- hypo-methylation of specific genes has also been reported in CVD. Through a gene ontology analysis, we found that the genes that have been reported to be differentially methylated in CVD are associated with pathways related to CVD pathogenesis, such as endothelial activation, oxidative stress and inflammation.

One of the most common adverse effects of mediastinal radiotherapy is radiation-induced CVD, which has the same presentation as atherosclerosis. Radiation-induced DNA damage, oxidative stress and inflammation are considered as major molecular drivers of radiation-induced CVD. Various therapeutic techniques have been employed to reduce the accidental irradiation of the heart during radiotherapy such as prone position radiotherapy, image- and dose- guided radiotherapy, IMRT, SBRT and DIBH. However, despite substantially reducing the dose of the accidental irradiation, radiation-induced cardiac events continue to occur. Furthermore, research is exploring protective agents which interfere with one or more of the identified pathophysiological mechanisms of radiation-induced CVD mainly through reducing chronic inflammation and oxidative stress [44,210,211]. In addition, non-traditional therapies against CVD are worth exploring

The late-onset aspect of radiation-induced CVD constitutes a diagnostic challenge to timely

initiation of cardioprotective therapy. Interestingly, radiation-induced DNA methylation alterations are notable immediately after irradiation and often persist long after it. Therefore, DNA methylation alterations in radiation-induced CVD could serve as novel, early biomarkers e.g. p16<sup>INK4a</sup>.

To the extent of our knowledge, no experimental research has directly investigated the correlation between radiation-induced DNA methylation alterations and resultant CVD. Previous research suggests the possibility of performing targeted DNA methylation [212] and demethylation [213,214]. This would allow studying the effect of prevention/induction of radiation-induced methylation alterations of specific genes. Subsequent effects on gene expression and induction of CVD pathophysiology could then serve as a means to test our theory. It is therefore our recommendation that future studies direct their attention to examining targeted radiation-induced DNA methylation alterations and their involvement in CVD pathogenesis. [194,215–271]

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