Fibrotic aortic valve disease after radiotherapy

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1. Introduction

The use of radiotherapy (RT) in the thoracic region has led to significant improvements in the treatment of breast cancer, malignant lymphomas, lung cancer, and other thoracic malignancies [1]. However, the introduction of this technique has led to a new range of cardiovascular disorders, caused by radiation injury to the heart and great vessels [1,2].

Patients treated for Hodgkins lymphoma (HL) until the mid-1990s received extended-field RT (35–45 Gy), with high radiation doses reaching the heart when areas above the diaphragm were involved. This is underlined among survivors of HL older than 60 years, of which almost 30% of the total excess number of deaths observed was due to heart disease [3]. Patients develop pericarditis, fibrosis, coronary artery disease, and valvular disease decades after RT. Radiation-associated valve disease (RAVD) is a late sequela after thoracic RT. Among patients treated with mediastinal irradiation for HL, there are statistically higher than expected rates of valvular heart disease [4]. Screening in HL survivors has reported that 42% of those given mediastinal irradiation had imaging evidence of valvular dysfunction after a median follow-up of 24 years [5].

Adverse effects of RT in patients with breast cancer are generally less pronounced as in HL, with patients treated for left-sided breast tumors receiving higher doses of radiation to the heart than patients with right-sided tumors [6,7]. This is emphasized by the increased risk of radiation-associated cardiac mortality in patients.
treated to the left breast or chest wall compared with those treated to the right side [6,8,9]. Extensively irradiated patients are more likely to develop radiation heart disease [10,11] and have increased risk for clinically significant RAVD [12]. Increased recognition of this risk has led to reduction in radiation doses and volumes when possible [13,14]. Risk of death from ischemic heart disease associated with radiation for breast cancer has substantially decreased over time [6]. Nevertheless, RT still results in cardiac injury if the disease affects the mediastinum.

Cardiac valves affected by RAVD exhibit fibrotic thickening and focal dystrophic calcification of the valvular leaflets [15,16]. The majority of patients that develop RAVD, however, show no symptoms of valvular dysfunction until two to three decades after RT [1]. It is suggested that regurgitation in the early phase of disease affects the mediastinum.

An in vitro study showed that radiation might induce an osteogenic phenotype in human AV interstitial cells [19]. Furthermore, research in cardiovascular tissue has shown that irradiation may activate various signaling cascades that induce tissue inflammation [20]. Pro-inflammatory cytokines can promote stiffening through valve interstitial cell activation and increased deposition of collagen and cytoskeletal contraction [21], which may lead to retraction and fibrosis of the valve leaflets.

The surgical pathology of RAVD has only been sparsely described in literature, and the most recent published series date back to the last century. Study of the valve histology after RT can provide insight into the mechanisms responsible for radiation-induced disease. We hypothesized that the exposure to radiation during RT is associated with AV inflammation, fibrosis, and calcification in patients with breast cancer and lymphoma.

We therefore conducted a histopathological study in patients with symptomatic stenotic AV disease who have received RT in the thoracic area to characterize and quantify inflammatory and extracellular matrix (ECM) changes at the time of aortic valve replacement (AVR).

2. Materials and methods

2.1. Specimen and data collection

Valve specimens were obtained from 43 patients referred to our center between 2008 and 2015 for surgical AV replacement because of symptomatic AV stenosis. A total of 28 consecutive patients had previously undergone thoracic or mediastinal radiation therapy for breast cancer (n = 22) or malignant lymphoma (n = 6), respectively. The patients with malignant lymphoma consist of patients with HL (n = 5) and a patient with non-HL (n = 1) who were grouped to increase statistical power and are from here on referred to as the lymphoma group. Fifteen patients with AV stenosis, who had never undergone thoracic or mediastinal RT, were included as reference material (controls). Controls were age-matched to patients treated with RT for breast cancer. Data of cardiovascular risk factors were extracted from patients’ records retrospectively. The clinical characteristics of the patients are given in Table 1. The criteria for the code of proper secondary use of human tissue in the Netherlands were met [22]. According to the Central Committee on Research involving Human Subjects (CCMO) and the Medical Ethics Review Committee of the Amsterdam UMC, this type of study does not require approval. All patients have approved to the secondary use of residual tissue for medical research.

2.2. Histology

After fixation in 10% neutral buffered formalin, representative parts at the centerline of the belly of all three leaflets were sampled for histology. When mineralization was present, the tissue was decalcified in 10% formic acid solution for 24 h. After the tissue was processed, the valves were sectioned at four µm in radial cross-sections, perpendicular to the free margin of the leaflets. Two observers, blinded to the groups, were used throughout all the analyses.

For histological analysis, sections were stained with hematoxylin-eosin (HE) for general morphology, Sirius Red (SR) for collagen, and Verhoeff's-Van Gieson (EvG) for elastin fibers. Using EvG we were able to differentiate between ECM and calcific nodules in decalcified tissue. All stainings were conducted within the same batch to avoid batch effects.

2.3. Immunohistochemistry

Immunohistochemical staining was performed to assess the presence of a late inflammatory cell response. Sections were stained for markers of T-lymphocytes (CD3; 1:200, Thermo Scientific, Waltham, MA, USA), B-lymphocytes (CD20; 1:100, Thermo Scientific, Waltham, MA, USA), and macrophages (CD68; 1:100, DakoCytomation, Glostrup, Denmark, and CD163; 1:200, Thermo Scientific, Waltham, MA, USA).

Immunostaining for CD3 and CD68 was performed after antigen retrieval by cooking in EDTA (pH 9.0) at 98 °C for 20 min. Antigen retrieval for CD20 and CD163 was performed in a citrate buffer (pH 6.0) at 98 °C for 20 min. Sections were stained with primary antibody for 1 h at RT and subsequently incubated in Poly-AP goat antirabbit (ImmuNoLogic, Duiven, the Netherlands) for CD3 and Poly-AP goat antimouse (ImmuNoLogic, Duiven, the Netherlands) for CD20, CD68, and CD163 for 30 min at RT. Secondary antibody binding was then visualized with Vector Red reagent (Vector Laboratories, Burlingame, CA, USA) and slides were counterstained with hematoxylin.

2.4. Image acquisition, processing, and analysis

Stained sections were automatically scanned with a Philips Ultra Fast Scanner 1.6 digital slide scanner (Philips Digital Pathology, Best, Netherlands). After scanning the sections, the tissue of the three leaflets per valve was completely captured at 2× magnification (pixel size is 5 µm) for histological analysis and at 40× magnification for IHC analysis (pixel size is 0.25 µm). The images were subsequently analyzed using ImageJ or Image Pro Premier v9.1 (Media Cybernetics, UK).

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AV</td>
<td>Aortic valve</td>
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<tr>
<td>AVR</td>
<td>Aortic valve replacement</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EvG</td>
<td>Verhoeff's-Van Gieson</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin-eosin</td>
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<tr>
<td>HL</td>
<td>Hodgkin lymphoma</td>
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<tr>
<td>RAVD</td>
<td>Radiation-associated valve disease</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>SR</td>
<td>Sirius Red</td>
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Breast cancer (n = 22) or malignant lymphoma (n = 6), respectively. The patients with malignant lymphoma consist of patients with HL (n = 5) and a patient with non-HL (n = 1) who were grouped to increase statistical power and are from here on referred to as the lymphoma group. Fifteen patients with AV stenosis, who had never undergone thoracic or mediastinal RT, were included as reference material (controls). Controls were age-matched to patients treated with RT for breast cancer. Data of cardiovascular risk factors were extracted from patients’ records retrospectively. The clinical characteristics of the patients are given in Table 1. The criteria for the code of proper secondary use of human tissue in the Netherlands were met [22]. According to the Central Committee on Research involving Human Subjects (CCMO) and the Medical Ethics Review Committee of the Amsterdam UMC, this type of study does not require approval. All patients have approved to the secondary use of residual tissue for medical research.
Valve thickness was determined in EVG sections by measuring the thickness of each leaflet at 5 sites in the free-floating belly area using Fiji v2.0 (ImageJ, National Institutes of Health) [23]. All measurements per leaflet were averaged. Cell density was calculated in 15 random 40× HE fields per valve by dividing the total number of counted nuclei in all fields by the total measured area in square mm.

Each 2× magnification field for histological analysis contains a complete section of the three leaflets per valve. SR stained area in the tissue was detected in ImageJ after applying image thresholds, and the number of red pixels was determined. The positively stained tissue was then divided by the total area of valve tissue on the slide to calculate the percentage collagen content of one section of three leaflets.

The amount of calcified tissue was quantified from EVG-stained slides using a color deconvolution algorithm with settings optimized for each slide, excluding all the calcified tissue from the digital image. The calcified tissue content per valve was then calculated as a percentage of the total area of valve tissue on the slide.

For IHC quantification, positive area for each marker per valve was measured in Image Pro Premier using computerized threshold segmentation of colors and expressed as the number of positive pixels per image. About 40–60 high power fields per valve were analyzed, encompassing one slide of the entire valve. To calculate the positive area percentage, the amount of total positive pixels was summed and divided by the total amount of analyzed pixels of all fields of a valve, resulting in the positive area percentage per valve.

2.5. Statistical analysis

Categorical variables are reported as number and percentage (%). Continuous variables are reported as the mean ± standard deviation (SD). Results were tested for Gaussian distribution using the D’Agostino–Pearson omnibus normality test; in the case of normal distribution in both groups, the unpaired T-test was performed, and nonnormally distributed data were tested using the Mann–Whitney U-test. One-Way ANOVA was used when three or more groups were compared. Categorical variables were analyzed by Fisher’s exact test. Correlation analysis was carried out using the Spearman correlation coefficients. All statistical analyses were performed using GraphPad Prism 6.01 for Windows (GraphPad Software, La Jolla, CA, USA). P ≤ .05 was considered statistically significant.

3. Results

3.1. Patient characteristics

All patients had stenotic tricuspid AVs. Mean age at AVR was lower after RT for lymphoma (62.3 ± 7.1) than after RT for breast cancer (73.7 ± 7.2, P = .003) and in the control group (75.0 ± 7.7 yrs, P = .005; Table 1). The lymphoma group was also exposed to RT at a much younger age (28.7 ± 9.7 vs 54.3 ± 12.3, P < .0001) than patients with breast cancer, as were left- compared with right-sided RT for breast cancer (45.2 ± 7.9 vs 58.8 ± 10.2 yrs, P = .01). The interval between RT and AVR was significantly longer after RT for lymphoma than after RT for breast cancer (32.0 ± 4.9 vs 18.6 ± 9.8 yrs, P = .005). Patients with breast cancer were treated with thoracic RT between 1976 and 2010 (median: 1995), whereas most patients with lymphoma were treated with mediastinal RT one decade earlier, between 1975 and 1988 (median: 1982; Supplemental Fig. S1).

3.2. Inflammatory markers

Expression of CD68+ macrophages was significantly lower in the breast cancer group, especially after left-sided RT, when compared with the controls (P = .003). No CD68+ giant cells were observed. CD3, CD20, and CD163 values were similar between the groups (Figs. 1 and 2A–D). We found a weak positive correlation between expression of CD3 T-cell and CD20 B-cell markers (r = .36, P < .013) and between CD68- and CD163-positive macrophages (r = .35, P = .02) when the groups are pooled (Supplemental Fig. S2). Within the breast cancer group, no significant differences were observed between site of radiation and expression of inflammatory cells (left or right; data not shown).

3.3. Cell density

Total AV cell density after RT for lymphoma (453 ± 117 cells/mm²) was markedly decreased when compared with the control (836 ± 238 cells/mm², P = .004) and breast cancer group (769 ± 275 cells/mm², P = .008; Fig. 2E). The age at RT and AVR positively correlated to cell density (r = .73, P < .0001 and r = .55, P = .003, respectively; Fig. 3A and B). Interval between RT and AVR negatively correlated to cell density (Supplemental Fig. S2).

3.4. Valve thickness

Valve thickness was similar between groups (P = .4; Fig. 2F). In the control valves, but not the RT valves, thickness negatively correlated to collagen content and positively to calcification amount (r = −.77, P = .005 and r = .86, P = .0004, respectively; Fig. 3C and D).

3.5. Calcification and fibrosis

AVs of patients exposed to RT for lymphoma contained more collagen and less calcified tissue than the control patients (45 ± 8 vs 24 ± 8%, P = .0005 and 12 ± 8 vs 26 ± 13%, P = .02, respectively; Figs. 1 and 2G–H). AVs of patients with breast cancer also contained...
Fig. 1. Histology and immunohistochemistry. Ctrl: Control; BCa: Breast cancer; Lym: Lymphoma. Scale bar SR and EvG = 500 μm; Scale bar CD3-CD163 = 50 μm.

Fig. 2. Quantitative analysis of valve (immuno)histology. Changes in inflammatory markers and valve histology between control, breast cancer RT, and HL RT-groups. A) CD3; B) CD20; C) CD68; D) CD163; E) cell density; F) valve thickness; G) and calcification; and H) collagen. Ctrl: Control; BCa: Breast cancer; Lym: Lymphoma. Box plots represent median with interquartile ranges ±1.5 times IQR. *P < .05, **P < .01, ***P < .001.
more collagen than control valves (32 ± 10 vs 24 ± 8%, \( P = .01 \)), but significantly less than after RT for lymphoma \( (P = .01) \). Valves after RT for breast cancer also contained significantly more calcification than after RT for lymphoma (20 ± 8 vs 12 ± 8%, \( P = .03 \)) and showed similar levels when compared with the control group (20 ± 8 vs 26 ± 13%, \( P = .16 \)). Within the breast cancer RT group, no differences were observed between left- or right-sided RT with respect to valvular damage (data not shown). In addition, the ratio of collagen to calcified tissue is significantly higher after RT for lymphoma compared with control and breast cancer RT \( (P = .001; \) Supplemental Fig. S2). Age at RT (RT groups pooled) and AVR negatively correlated to valve collagen content \( (r = -0.54, P = .004 \) and \( r = -0.49, P = .008, \) respectively; Fig. 3E and F). Age at RT, but not at AVR, strongly correlated to valve calcified tissue content \( (r = 0.60, P = .003 \) and \( r = 0.35, P = .087, \) respectively; Fig. 3E and F). Presence of collagen or calcified tissue did not correlate to observed cell density (Supplemental Fig. S2).

3.6. Chemotherapy

A total 6/22 (27%) patients with breast cancer, and 2/6 (33%) patients with lymphoma received chemotherapy besides RT. On average, patients receiving combination therapy were younger when treated for their malignancy, albeit not significantly (49 ± 10 vs 57 ± 13 yrs, \( P = .28 \)). Patients with breast cancer who received combined RT + chemotherapy also presented for AVR at a younger age than patients who received RT alone (69 ± 5 vs 76 ± 7 yrs, \( P = .014 \)), while the interval between RT and AVR was similar \( (P = .53; \) Supplemental Fig. S3). No other significant differences between groups among all patient characteristics and results were observed (not shown).

4. Discussion

The present study was aimed to gain insight into the late inflammatory, fibrotic, and calcific changes of the irradiated AV. The results show that RT for lymphoma and breast cancer does not result in chronic inflammatory infiltration in human AVs when compared with nonirradiated stenotic valves. High dose radiation at a young age (patients with lymphoma) results in fibrotic AV stenosis with a decreased total cell density. As patients with breast cancer were older at the time of radiation and AVR, their valves show a mixed-type AV stenosis with significantly more calcification.

Patients with lymphoma in our group were exposed to extended-field RT between 1975 and 1988 (median: 1982) at the average age of 29 years, significantly earlier and younger than the 54 years of the irradiated breast cancer patients that were treated between 1976 and 2010 (median: 1995). Patients treated for lymphoma developed symptomatic AV disease three decades after initial RT, compared with one to two decades on average after RT for breast cancer. Patients with lymphoma were elected for AVR at the average age of 62 years, whereas irradiated breast cancer patients and our nonirradiated control group were 10 years older (73 and 74 years, respectively; slightly above the average age at AVR in the general Dutch population between 2010 and 2014 [24]).

These numbers agree with what is known in current literature about lymphoma and the cardiac effects of RT. Patients with lymphoma are generally young adults when treated and have increased risk of cardiac death [1,25]. Our patients with lymphoma underwent mantle field RT three to four decades earlier, which means that these patients were often prescribed higher doses than are currently in use (35–45 Gy) [28]. Several studies concluded that there is an increased risk for cardiac death and clinically significant RAVD after RT for lymphoma for radiation doses of >30 Gy [11,12,25]. Mediastinal radiation of ≤30 Gy increased the 30-year risk of valve disease by only about 1.4% [12].

These studies suggest that high radiation dose to the heart valves increase the risk for clinically significant RAVD with a possible threshold dose of about 25–30 Gy.

Substantial changes in irradiation techniques have occurred over the last 10–20 years, reducing cardiac morbidity and mortality.
However, patients with lymphoma in the Netherlands are still treated with high-dose (30–36 Gy) mediastinal RT, and these patients may thus have an increased risk of RAVD over the next 2–3 decades. Planned new guidelines for treatment of patients with lymphoma will include lower doses (20 Gy), but these will only be applicable in a small specific patient group. It is, however, unclear to what radiation doses the valves included in the present study were exposed. We assumed, according to their historical treatment regimes, that the patients with lymphoma in our study were likely exposed to radiation doses of >30 Gy, whereas patients with breast cancer received far lower doses during RT. We observed a decrease in cell density in the AV after RT for lymphoma. This may be caused by the high dose received during historic RT for lymphoma. As previously stated, there seems to be a threshold at >30 Gy after which there is an increased risk of cardiac sequelae after RT. This threshold may also be true at a cellular level, with doses of >30 Gy inducing a certain effect leading to cell loss. In contrast, heart valves of patients with breast cancer generally receive lower doses during RT and may not reach this threshold and are able to recover. As the adult AV is thought to have low cellular turnover with minimum proliferative capacity [27], cell death due to radiotoxicity may lead to permanent cell loss. Commonly, a progressive age-associated decrease in cell number is seen from fetal to children's and adult valves [27], which may be associated with valve degeneration.

Patients treated for breast cancer are generally older than patients with lymphoma and are, because of age alone, already at risk for cardiovascular disease. Patients treated for left-sided breast tumors receive slightly higher doses of radiation to the heart than patients with right-sided tumors [6,7]. This is underlined by the increased risk of radiation-associated cardiac mortality [8,9]. Left-sided breast cancer is treated with angled fields, which may include only the cardiac apex. The base of the heart, including the heart valves, is minimally exposed. Involvement of the internal mammary chain, however, may increase cardiac exposure [28]. We observed no difference between left- or right-sided RT regarding AV cellular composition and ECM pathological changes.

Besides RT, several studies have observed an increased risk of myocardial injury and valvular disease after chemotherapy, particularly with anthracyclines and alkylating agents, which are still commonly used in the clinic [29–32]. One of these studies showed increased AV degeneration after chemotherapy alone without RT [31]. We did not observe more severe valve disease in the patients that underwent chemotherapy besides RT. These patients, however, were significantly younger at AVR; this is likely because they were also younger at the time of RT, as the interval between RT and AVR remained the same.

In our study group, AVs of patients with lymphoma appear more fibrotic than calcified. This may be because they were relatively younger, and age is associated with calcific AV disease [33]. On the other hand, they received more extensive RT and are more likely to develop radiation heart disease [10]. If we assume a gradient from low to high RT dose in Fig. 2H, controls would be “no radiation”, breast cancer would be “low dose”, and lymphoma would be “high received dose”. A positive trend is visible for collagen content and radiation dose, as if valvular fibrosis was dose-dependent. An opposite trend is visible for calcification but differences were less pronounced, and no difference was observed in calcification between RT for breast cancer and controls. These observations could be explained by the age of the different groups at RT or AVR. All valves pooled, age at RT and AVR negatively correlated to collagen content, and age at RT positively correlated to the amount of calcified tissue. However, valves after breast cancer RT are of similar age as controls and still contained significantly more collagen. This may suggest some fibrotic effect of RT in the AV, especially when administered at high dose and young age. These findings are in line with several previous studies in which high dose and young age were predicted as risk factors for cardiac and valve disease [1,2,3,6,7,29,30,34].

4.1. Conclusion

Young patients who receive high-dose radiation to the mediastinum develop early functional valvular abnormalities that may be associated with fibrosis and cell loss. The active presence of inflammatory cells is likely limited to the acute phase after RT while secondary effects such as ECM changes persist. Our findings imply a possible dose-dependent effect of RT on AV fibrosis, with a hypothetical threshold at ~30 Gy above which irreversible cellular damage may be induced. Patients with breast cancer were older at the time of RT and AVR, and their valves show a mixed type with both fibrosis and calcification, more typical to calcific AV stenosis. Although being purely of an exploratory nature, our study emphasizes the unwanted fibrotic effects of high-dose mediastinal RT on the AV and underlines the importance of field reduction and lowering radiation dose to further decrease cardiac exposure and premature RAVD. Additional investigation is needed to define the dose-dependent mechanistic linkage between cell loss, inflammation, fibrosis, and other radiation-induced pathologies in RAVD and how these changes are initiated and maintained in time.

Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carpath.2019.107176.
References