



Defining Fingerprint Pattern of Cardiotoxicity and Targeting Specific Underlying Mechanisms of Cardiotoxicity Could be the Way for Successful Cardioprotective Measures in Paediatric Cancer Patients Treated with Anthracyclines

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Review

Anthracycline Chemotherapy are administered to more than 50% of children with cancer and up to 60% of those treated with an anthracycline will develop echocardiographic abnormalities [1]. Compared to their siblings, childhood cancer survivors (CCSs) have a 15-fold increased risk of developing congestive heart failure (CHF) and mortality rate in those with CHF approaches 50% [2]. Once anthracycline-induced cardiomyopathy develops, it is commonly progressive, with no definitive treatment other than implantation of a left ventricular assist device and/or heart transplantation [3,4]. Cardiac disease is the third leading cause of premature death in CCSs after cancer recurrence and second malignancies [5]. Early medical intervention can improve parameters of cardiotoxicity; the most feared complication of anthracycline chemotherapy [6]. Monitoring of cardiotoxicity involves using echocardiographic measures of systolic function {left ventricular (LV) ejection fraction (EF) or shortening fraction (SF)} or clinical cardiac disease manifestations. These parameters are frequently normal early on even when biopsy demonstrate significant evidence of myocardial damage such as apoptosis and interstitial fibrosis [1,7]. Moreover, heart failure may not occur for years (or even decades) after anthracycline exposure and it can exist alongside normal EF if the end diastolic volume (EDV) is too small [8]. Therefore, there is a need to use more sensitive and reliable imaging techniques, genetic testing and biomarkers for earlier detection of subclinical or mild asymptomatic treatment-induced cardiac toxicity before it progresses to HF. This will allow for identifying survivors at greatest risk for progressive cardiac deterioration who require early medical intervention.

Some novel and more sensitive imaging techniques have been shown in adults to detect early changes in cardiac size, shape,

structure, and function (i.e. remodelling) prior to the decrease in LVEF [1,9-11]. Examples of these techniques are "Speckle Tracking Echocardiography" (STE) that analyses the motion of tissues in the heart, "Strain Rate Imaging Echocardiography" for measuring regional or global deformation of the myocardium, "Tissue Doppler Echocardiography" that measures LV Posterior Wall Thickness and LV Thickness to Dimension Ratio and "Cardiac Magnetic Resonance" (CMR) using T1 relaxometry based approaches (T1 mapping), to measure myocardial extracellular volume (ECV) which correlates with the degree of cardiac fibrosis. There are still limited data in children on the utility of these novel imaging techniques.

Evidence of the role of genetic factors is rapidly expanding our knowledge and ability to predict and manage anthracycline-induced cardiotoxicity. Some genetic variants have been linked with anthracycline-induced cardiotoxicity in children [12]. A recent genome-wide association study (GWAS) reported that a non-synonymous coding variant (rs2229774, S427L) in retinoic acid receptor gamma (RARG) is associated with anthracycline-induced cardiotoxicity in children. This association was replicated in European, African, East Asian, Hispanic and Aboriginal Canadian patient populations. The RARG rs2229774 variant has been shown to alter RARG function leading to a reduced repression of the key anthracycline-induced cardiotoxicity genetic determinant, DNA topoisomerase 2-beta (TOP2B) [13]. Two variants in the solute carrier (SLC) transporter SLC28A3 (rs7853758, rs885004) have shown consistent associations with doxorubicin and daunorubicin-induced cardiotoxicity in three independent well characterized paediatric cohorts [14]. The known function of the SLC super family as drug transporters and the reported transport

of anthracyclines by SLC transporters [15] provides biological support for these genetic associations. A synonymous coding variant (rs17863783, V209V) in UDP-glucuronosyltransferase family 1A, isoform 6 (UGT1A6) showed evidence for an association with an increased risk of anthracycline-induced cardiotoxicity in three independent paediatric patient populations [14]. Reduced UGT1A6-mediated glucuronidation of anthracycline metabolites may lead to accumulation of toxic metabolites in patients carrying UGT1A6, resulting in the observed increased risk of anthracycline-induced cardiotoxicity [16]. Reported results about other genetic variants' associations with anthracycline-induced cardiotoxicity were conflicting and could not be replicated in other studies. Taken together, RARG rs2229774, SLC28A3 rs7853758 and UGT1A6 rs17863783 variants currently have the strongest and the most consistent evidence for association with anthracycline-induced cardiotoxicity in children. Therefore, a moderate clinical practice recommendation was made based on reduced confidence scientific evidence and expert opinion; benefits likely to outweigh risks, to do pharmacogenomic testing in all childhood cancer patients with an indication for doxorubicin or daunorubicin therapy for RARG rs2229774, SLC28A3 rs7853758 and UGT1A6*4 rs17863783 variants [2]. The goal of these recommendations is to provide optimal clinical care through guidance on the use of genetic information on risk of adverse effects to reduce the incidence of cardiotoxicity and CHF in children receiving anthracycline chemotherapy for cancer treatment. Worth mentioning is that genetic testing is currently not recommended in adult patients and in children receiving other types of anthracyclines. It is also to be noted that CYP3A5 rs4646450, ABCC2 rs3740066, NQO1 rs1043470 and SLC22A6 rs6591722 variants are reported to be associated with anthracycline-induced cardiotoxicity in children [17].

A number of cardiovascular disease-related biomarkers have been discovered and utilized in clinical settings. According to the American Association for Clinical Chemistry (AACC), common cardiac biomarkers extensively used are cardiac troponin and creatine kinase (CK). Cardiac troponins as proteins that form actin/myosin bridges in the striated muscles are the gold standard biomarker that measure any scenario involving myocyte injury. Troponin T (TnT) has been shown to be elevated in children treated with anthracyclines [18]. However, studies have yet to reveal quantitative trends in troponin levels that specifically predict late cardiac outcomes, and thus troponins are insufficient to guide cardioprotective intervention [19]. The biologically active brain natriuretic peptide (BNP) and inactive N-terminal pro-brain natriuretic peptide (NT-pro-BNP), secreted from the ventricles of the heart in response to increased ventricular volume and pressure, are also predictive of heart failure and considered as valuable biomarkers of chemotherapy-induced cardiotoxicity. Measurement of natriuretic peptides as indicators of myocyte stress is commonly used in hospitals worldwide [20]. The NT-pro-BNP in particular has been demonstrated to be elevated in children treated with anthracycline [18]. Some other biomarkers have been associated with cardiac fibrosis and extracellular remodeling. Biomarkers of collagen metabolism; C-terminal propeptide (PICP) and C-terminal

telopeptide of type 1 collagen (CTx) as well as the N-terminal peptide of procollagen type III identify the presence of fibrosis in HF [21]. Both circulating matrix metalloproteinases (MMPs) and TIMPs (endogenous tissue inhibitors of metalloproteinases, which comprise a family of four protease inhibitors) are involved in cardiac remodeling; MMP2, MMP3, and MMP9 seem to play a role in the development of HF. Increased MMP-3 and MMP-9 concentrations are associated with higher mortality rates in patients with HF with reduced Ejection Fraction [22]. Galectin (gal)-3 (a member of the beta-galactoside-binding protein family and encoded by the LGALS3 gene) is also being used in clinical practice to detect and predict HF [23] since it has been demonstrated to be associated with cardiac remodeling/fibrosis/inflammation [24,25]. Similarly, ST2 is a member of the interleukin-1 receptor family biomarker and circulating soluble ST2 concentrations are believed to reflect cardiovascular stress and fibrosis [23]. Nevertheless, laboratory biomarkers of cardiac remodeling and fibrosis are non-cardiospecific biomarkers and so their usefulness has to be better established in the presence of comorbidities such as diabetes, chronic systemic inflammatory diseases, and renal and liver diseases. It is important to classify HF biomarkers according to mechanisms that involve myocyte injury and apoptosis, myocardial stress/stretch, inflammation and fibrosis, extracellular matrix remodeling, neurohumoral activation, oxidative stress, and extra-cardiac involvement [23]. Identifying patients with more ventricular fibrosis from those with more neurohumoral activation, or patients with predominant inflammation or apoptosis for example will allow for a tailored therapy reflecting the characteristics of each patient.

With the advancements of genome-wide analyses and RNA-sequencing technologies, new components of the genome have been discovered, including noncoding RNAs which are regions of the genome that do not code for proteins, but can regulate the function of genes and therefore affect physiological and pathological processes. These noncoding RNAs can be classified into different types according to their function, length and structure. Three types; microRNAs (miRNAs or miRs; 19–23 bp), long noncoding RNAs (lncRNAs; >200 bp and linear), and circular noncoding RNAs (circRNAs; >200 bp and circular), are considered promising biomarkers of cardiovascular disease and they are important not only in diagnosis but also in the treatment, and prognosis of cardiovascular diseases such as myocardial infarction, atherosclerosis, cardiomyopathy and cardiac fibrosis [19,26,27]. Cardiomyopathy, cardiac apoptosis, fibrosis and remodeling are of particular importance in anthracycline-induced cardiotoxicity. CircRNA_000203 and CircRNA_010567 have been shown to regulate cardiac fibrosis by sponging miR-26b-5p and miR-141, respectively causing loss of their function phenotypes. It is also known that CircRNA_000203 activates cardiac remodeling and that CircRNA_010567 increases the rate of myocardial fibrosis by inhibiting miR functions [28,29]. The heart-related CircRNA HRCR can also sponge the deleterious miR-223, leading to cardioprotection against heart failure [30]. A more recent study showed that circRNA MFACR regulates mitochondrial fission and apoptosis in the heart by downregulating miR-652-3p and subsequently upregulating mitochondrial protein 18 kDa

(MTP18) [31]. Cardiomyopathy, including hypertrophic and dilated cardiomyopathy can be induced as part of anthracycline-induced cardiotoxicity. Ten exonic calcium/calmodulin-dependent protein kinase type II delta (camk2d) and titin circRNAs were also found to be associated with cardiomyopathy in heart samples from HCM and DCM patients. Camk2d circRNAs were downregulated in both HCM and DCM patients, while titin circRNAs were downregulated only in DCM [32]. Hypertrophic cardiomyopathy (HCM) occurs when the LV myocardium becomes thicker than normal, leading to incomplete filling at diastole eventually leading to HF and death. Dilated cardiomyopathy (DCM) occurs when LV or RV functions are impaired, leading to ventricular dilation, HF and death.

Heart-related circRNA (HRCR) was reported to have protective effects against myocardial hypertrophy and heart failure where it interacts with miR-223, retaining it in the cytoplasm, and inhibiting the pro-hypertrophic activity of miR-223 [30]. Circular RNAs are more stable and abundant in extracellular fluids than miRNAs and lncRNAs. They are also present in blood, plasma and extracellular vesicles and therefore most components of the blood (eg, peripheral blood mononuclear cells, exosomes, platelets, plasma and serum) can be used for biomarker studies [25,33]. In addition, certain circRNAs; Circ_0000615 (MICRA), Circ_0000005, Circ_0000673, Circ_0000585, Circ_0000816, Circ_0000817, Circ_0000917, Circ_0001423, Circ_0000540, Circ_0001844, Circ_0000994 and CircNPPA, can be used as biomarkers for HF and myocardial hypertrophy since patents exist demonstrating that they are dysregulated in these diseases [34]. Circulating microRNAs (miRNAs) are also considered a promising biomarkers of cardiovascular disease with high stability in plasma, tissue-or cell-specific distribution, and ease of quantitative measurement [35]. They are promising biomarkers specifically for early detection of anthracycline (AC)-induced cardiotoxicity that may allow for cardio preventive intervention before irreversible damage [19]. Such miRNAs have been associated with cardiac fibrosis and are suggested to be used in diagnosis of HF [23]. Cardiac-related plasma miRNAs are dysregulated following anthracyclines. Plasma miR-29b and -499 are acutely elevated post-anthracycline, with dose response relationships observed with anthracycline dose and markers of cardiac injury. Normalized miR-1, miR-29b, and miR-499 were significantly upregulated from baseline post-anthracycline at multiple time points compared to controls receiving noncardiotoxic chemotherapy [19].

The role of genetic variants and noncoding RNA associated with anthracycline-induced cardiotoxicity in children is under investigated and has not been specifically validated in specific types of cancer. Collaborative approaches between basic scientists and clinicians are required to validate and integrate reliable genetic tests and specific biomarkers into clinical practice for the purpose of development of diagnostic and therapeutic modalities for such diseases. The cardiotoxic mechanisms of anthracyclines are collectively believed to involve the dual pathways of reactive oxidation species and topoisomerase 2-beta and a final common pathway of calcium overload, lipid peroxidation and mitochondrial dysfunction. Nevertheless, HF biomarkers could reflect mechanisms that involve myocyte injury and apoptosis, myocardial stress/

stretch, fibrosis, extracellular matrix remodeling, neurohumoral activation, oxidative stress, and extra-cardiac involvement. Therefore, identifying patients with more ventricular fibrosis from those with more neurohumoral activation, or patients with predominant inflammation or apoptosis for example will allow for a tailored therapy reflecting the characteristics of each patient [23].

Specific cardioprotective medications like mineralocorticoid receptor antagonist (MRA) may be considered in case of rise of biomarkers of collagen metabolism, galactin-3 and ST2 reflecting fibrosis after excluding other etiologies since such biomarkers may not be cardio-specific. Clinical trials have demonstrated that MRA therapy reduces morbidity and mortality in patients with HF due to left ventricular systolic dysfunction and is of therapeutic value in HF patients with preserved ejection fraction. Such beneficial effects of MRAs were explained by the effect of MRAs in preventing many of the maladaptive effects of aldosterone on the cardiovascular system including sodium and fluid retention, myocardial fibrosis, vascular stiffening, endothelial dysfunction, catecholamine release and stimulation of cardiac arrhythmias [36].

One cardiopreventive strategy in subclinical cardiomyopathy due to anthracycline treatment is to target mitochondrial oxidative stress with the iron chelator; dexrazoxane as it is believed to be of value mainly to augment antioxidant defense mechanisms. Elamipretide; a promising mitochondrially-targeted tetrapeptide that appears to reduce the production of toxic reactive oxygen species and stabilize cardiolipin; an important component of the inner mitochondrial membrane that is essential for the optimal function of numerous enzymes involved in mitochondrial energy metabolism. Several phase 2 clinical trials with Elamipretide in HF patients are underway [37,38]. Nevertheless, the biomarkers for mitochondria microcirculation and metabolic abnormalities as one mechanism for anthracycline-induced HF are missing.

Biomarkers reflecting myocardial inflammation and oxidative stress such as cytokines, C-reactive protein (CRP), myeloperoxidase (MPO), growth/differentiation factor-15 (GDF-15) and pentraxin 3 acute phase proteins (APPs) could help in directing cardioprotective strategies to the use of certain medications. Metformin has anti-inflammatory and antioxidative properties and may reduce the risk and incidence of HF and mortality in diabetic patients, while improving survival rates up to 2 years in those with HF. Nevertheless, the recently reported cardio preventive effects of metformin may not be universal to all forms of HF and may require AMP-activated protein kinase activation or ATP depletion and it is contraindicated in patients with severe renal or hepatic impairment, because of the risk of lactic acidosis [39]. Statins are also suggested to have therapeutic value in patients with HF since they reduce lipid concentration, exhibit pleiotropic activity, improve endothelial function, increase nitric oxide synthesis, exert anti-inflammatory and antioxidant effects, inhibit neurohumoral activation, and benefit myocardial reconstruction. The beneficial value of statins in patients with HF though is controversial. Although a comprehensive, well-conducted meta-analysis study investigated whether statin therapy had an effect on major HF outcomes such as hospitalization and death and the results demonstrated a significant

effect of statins on HF hospitalizations, several limitations involving the participant data and nature of statin used in the analyzed trials raise questions about the inferences that can be drawn from the study results [40].

Cardioprotective strategies targeting myocyte injury and apoptosis may be of value when there is a rise in high sensitivity troponin T (hs-cTnT) as a biomarker that could be elevated in children treated with anthracyclines indicating myocyte injury, cardiomyocyte apoptosis, and/or myofibril degradation. Angiotensin Receptor-Nephrilysin Inhibitor (ARNi) medication is a combination of angiotensin receptor blocker and neprilysin (a membrane metallo-endopeptidase) inhibitor recommended for use as a replacement for an ACE inhibitor or an angiotensin receptor blocker in people with heart failure with reduced ejection fraction. The benefit of this combined medication appeared to be related to improvement in rates of progressive pump failure or sudden cardiac death/apoptosis [41]. Another medication; ivabradine was demonstrated in several experimental studies to exhibit cardioprotective effect through anti-apoptotic mechanisms and erythropoietin was also reported to prevent the apoptosis of vascular endothelial cells, and cardiomyocytes [42-44]. Knowing the role that beta adrenergic receptor stimulation play in cardiac injury and apoptosis, some beta blockers were also shown to provide significant cardioprotective effect against cardiomyocyte injury mediated through multiple mechanisms, including the inhibition of cardiomyocyte apoptosis [45,46]. Calcium channel blockers may also have a value as cardiopreventive in HF knowing that in children treated with anythracyclines, reactive oxygen species (ROS) can induce autophagy (a process of intracellular components' degradation) in cardiac myocytes, which in turn facilitates development of pathological hypertrophy and participates in the pathogenesis of HF, and that calcium overload has important role in maintaining autophagy at a higher level [47].

Other potential biomarkers exist indicating neurohumoral activation and/or myocardial hypertrophy such as renin, angiotensin II, aldosterone, plasma renin activity (PRA), neurotrimin (a protein related to the family of immunoglobulins), urocortin 1 (a vasoactive peptide belonging to the corticotrophin-releasing factor peptide family), fibroblast growth factor 23 (FGF23), arginine vasopressin (AVP), endothelin 1 (a potent endothelium-derived peptide that regulates cardiovascular function much like angiotensin II and catecholamines), copeptin and midregional part of proadrenomedullin (MR-proADM). The latter two are stable by-products of vasopressin and adrenomedullin. Such biomarkers may direct to the potential cardioprotective effect of certain medications such as ACE inhibitors, angiotensin receptor blockers (ARB), angiotensin receptor-nephrilysin inhibitors (ARNi), mineralocorticoid receptor antagonist (MRA), and beta blockers. Other medications of potential value in such cases include serelaxin (RLX); a recombinant human relaxin-2 with vasodilator effect exerted mainly via multiple NO-mediated mechanisms, and tolvaptan (TLV); an oral vasopressin V2-receptor antagonist. Both serelaxin and tolvaptan appeared to counteract the activation of neurohormonal systems such as renin-angiotensin-aldosterone

system (RAAS) among other pathophysiological processes involved in HF [48,49]. The antihypertrophic therapy also extends beyond blocking the classical β -adrenergic and RAAS-dependent signalling cascades, where other new therapies such as third generation aldosterone antagonists (PF-03882845 and BAY94-8862), biased angiotensin II receptor agonists and microRNA-based therapy are under evaluation in addition to approaches to stimulate cGMP-dependent protective signalling, and to inhibit Ca²⁺-mediated transcriptional regulation and PI3 kinase gene transfer [50,51]. Another very promising cardioprotective strategy is through endothelin-1 antagonism since the pre-treatment of endothelin-1 receptor antagonist; bosentan prior to doxorubicin has been shown to be cardioprotective although the exact mechanism of such effect is yet to be finalized [52].

Some biomarkers of kidney dysfunction, such as cystatin C, neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule -1 (KIM-1), and N-acetyl- β -D-glucosaminidase are of importance in HF knowing that heart and kidney are interdependent organs where the heart is directly dependent of the regulation of salt and water content of the body by the kidneys and also the kidneys are directly dependent of blood flow and pressure generated by the heart. Controlling the progression of the renal disease in such cases is the way to reduce cardiovascular risk.

Finally, it is essential to recognize the multifactorial processes that lead to the development of cardiovascular disease in children treated for malignancies. Clinically significant cardiac toxicity from anthracyclines is unpredictable in individuals, even with similar cumulative doses and clinical conditions and there is a wide variation in individual cardiac toxicity pattern from anthracyclines. Therefore, as an opinion, it is important to apply a tailored preventive medication strategy for children with cancer who are at risk of developing CHF as a result of anthracycline treatment. Identifying a fingerprint pattern of cardiotoxicity for each case within a certain type of cancer together with targeting specific underlying mechanisms of cardiotoxicity is the way for successful cardioprotective measures. The fingerprint pattern of cardiotoxicity involves different factors such as whether clinically symptomatic heart failure occurred or only asymptomatic fall in LVEF, whether there is a rise in cardiac and or extra-cardiac biomarkers reflecting mechanisms of HF, the cumulative dose of anthracycline used, the presence of other cardiovascular risk factors, the age of treatment, the imaging pattern of subclinical cardiac dysfunction, the presence of genetic variants and type of therapeutic interventions.

Toward achieving that goal, laws, regulations and ethical guidelines should be followed in conducting international multiphase prospective cohort studies of children receiving anthracyclines for one type of malignancy to observe for the relationship among genomic variations, biomarkers and echocardiographic remodeling/dysfunction parameters and their role in diagnosis & prognosis of anthracycline-induced cardiotoxicity.

In the first phase (acute/subacute and early onset chronic progressive cardiotoxicity cohort), the relationship among genetic predictors of anthracycline susceptibility, biomarkers of early

cardiac damage, and imaging parameters of subclinical cardiac dysfunction could be investigated. Whether these parameters can predict which patients will demonstrate evidence of persistent or progressive cardiac damage at the 12 months follow-up from their last cycle of anthracycline chemotherapy will also be assessed.

For that aim the following procedures could be followed

At baseline (before anthracycline administration)

- a. Clinical data, family/medical history, demographics, and data of concomitant medications are collected.
- b. Saliva sample is collected for DNA extraction and genomic analysis. Exome sequencing is performed where sequencing is performed only for the protein-coding genes in the genome (selection of only the subset of DNA that encodes proteins; exons). The top-ranked genes that are enriched for variants (mutant forms) and are deemed biologically relevant will undergo targeted sequencing to determine nucleotide order. Genes in pathways related to anthracycline absorption, distribution, metabolism, and excretion, and genes important in cardiac response to injury will be prioritized in the analysis (much lower cost than whole-genome sequencing). Of importance is the detection of RARGrs2229774 (topoisomerase-2 β expression), SLC28A3 rs7853758 (drug transport), and UGT1A6 rs17863783 (drug metabolism) variants which have the strongest and the most consistent evidence for association with anthracycline-induced cardiotoxicity in children.
- c. LVEF in addition to early cardiac remodeling parameters that precede global dysfunction; LV Posterior Wall Thickness (LVPWT) and LV Thickness to Dimension Ratio (LVTDR) will be measured by Tissue Doppler Echocardiography as markers of early cardiac injury that can identify patients who are at risk for progressive cardiac dysfunction later in life. Also, mean circumferential/ longitudinal strain will be measured using Speckle Tracking ECHO.
- d. Serum levels of some of the following biomarkers/mediators of cardiac disease could be conducted:
 - a. High sensitivity troponin T (hs-cTnT) as an indicator of myocyte injury (it may also indicate cardiomyocyte apoptosis, myofibril degradation and predicts cardiotoxicity) [Immunoassay, ELISA].
 - b. N-terminal pro-brain natriuretic peptide (NT-pro-BNP) as an indicator for myocyte stress [Automated NT-proBNP assay, ELISA].
 - c. circRNAs; Circ_0000615 (MICRA), Circ_0000005, Circ_0000673, Circ_0000585, Circ_0000816, Circ_0000817, Circ_0000917, Circ_0001423, Circ_0000540, Circ_0001844, Circ_0000994 and CircNPPA, as indicators for HF and myocardial hypertrophy [RT-qPCR analysis using divergent primers for detection, validation, and quantification of circRNAs].
 - d. CircRNA_000203 and CircRNA_010567 as regulators of cardiac fibrosis [RT-qPCR].

- e. The heart-related CircRNA HRCR as cardio-protective against myocardial hypertrophy in HF [RT-qPCR].
- f. circRNA MFACR as a regulator of mitochondrial fission and apoptosis in the heart [RT-qPCR].
- g. calcium/calmodulin-dependent protein kinase type II delta (camk2d) circRNA and titin circRNAs as regulators of cardiomyopathy [RT-qPCR].
- h. miRNA-1, miRNA-29b, and miRNA-499 as biomarkers or mediators of cardiovascular disease which were reported to be specifically associated with cardiac fibrosis [possible techniques: northern blotting, microarray, in situ hybridization (ISH), and nucleic acid amplification techniques, including polymerase chain reaction (PCR), ligase chain reaction (LCR), rolling circle amplification (RCA), exponential isothermal amplification reaction (EXPAR), nuclease-assisted amplification, nanomaterial-based detection, s droplet digital PCR (ddPCR), electrochemiluminescence (ECL), surface-enhanced Raman spectroscopy (SERS) and mass spectrometry (MS)].
- i. Biomarkers of collagen metabolism proposed as potential useful tools to improve diagnosis, prognosis, and therapy in cardiac dysfunction that develops to HF; carboxyterminal propeptide of type I collagen (PICP), carboxyterminal telopeptide region of type I collagen (CTX), and N-terminal peptide of procollagen type III as an indicator of fibrosis in HF [Enzyme-linked immunosorbent assays, and double antibody radioimmunoassays]
- j. Circulating matrix metalloproteinases (MMPs; MMP2, MMP3, and MMP9) and TIMPs as indicators of cardiac remodeling (2-site sandwich enzyme-linked immunosorbent assays in serum).
- k. Galectin (gal)-3 which is being used in clinical practice to detect cardiac fibrosis [ELISA].
- l. Pentraxin 3 acute phase protein (APPs) as an indicator of inflammation/oxidative stress [ELISA].
- m. Myeloperoxidase (MPO) (ELISA)
- n. Insulin-like growth factor binding protein 7 (IGFBP7) as an indicator of stress / injury (Chemiluminescence- linked immunoassay).

Twenty four hours after each anthracycline dose

- i. LVEF in addition to LV Posterior Wall Thickness and LV Thickness to Dimension Ratio (using Tissue Doppler Echocardiography) could be measured. Also, mean circumferential/ longitudinal strain will be measured using Speckle Tracking ECHO.
- ii. Serum level of some of the above-mentioned biomarkers/mediators could be determined.

Three months after the final anthracycline dose

- i. Serum level of some of the above-mentioned biomarkers/mediators could be determined.

Twelve months after the final anthracycline dose

- a. LVEF, LV Posterior Wall Thickness, LV Thickness to Dimension Ratio and mean circumferential/ longitudinal strain could be measured.
- b. Serum level of some of the above-mentioned biomarkers/ mediators could be determined.

In the second phase (late-onset chronic progressive cardiotoxicity cohort), the relationship among genetic predictors of anthracycline susceptibility, biomarkers of early cardiac remodeling /dysfunction, and imaging parameters of subclinical cardiac dysfunction could be investigated. Whether these parameters can predict which patients will demonstrate evidence of chronic progressive cardiac damage 3 to 10 years after their last cycle of anthracycline chemotherapy will also be assessed in the same patients followed up in the first phase.

For that aim the following procedures could be followed**At baseline (three years after the last anthracycline dose)**

- i. Clinical data, medical history, and data of concomitant medications are collected.
- ii. LVEF, LV Posterior Wall Thickness, LV Thickness to Dimension Ratio and mean circumferential/ longitudinal strain could be measured.
- iii. Serum level of some of the above-mentioned biomarkers/ mediators could be determined.

The ECHO and biomarkers' assessment mentioned above could be performed every year during the follow up period to reach 10 years after their last cycle of anthracycline chemotherapy.

Thus, the primary outcome measurement is the presence of 1 or more of the following at 3 or 12 months after the last anthracycline dose in the first phase, or at any study time point in the second phase;

- i. Reduced LV EF (<55%) or a drop in LV EF of $\geq 10\%$ over serial echocardiogram.
- ii. Mean circumferential strain measurement $> -15\%$ or mean longitudinal strain $> -18\%$.
- iii. Reduction in LVPWT or LVTDR z-score by ≥ 1 standard deviation compared to baseline (Z-score is the number of standard deviations from the mean a data point is). It will be measured and calculated as recommended by the paediatric quantification guidelines issued by the American Society of Echocardiography.
- iv. Symptomatic heart failure graded using New York Heart Association (NYHA) classification (or Ross heart failure class 2 in infants <2 years old).

Special Emphasis could be Devoted to the Following Objectives

- A. Determining whether myocardial strain measurements (Mean circumferential/ longitudinal strain) are observed

after acute anthracycline exposure. These will be analysed to evaluate their usefulness for the early detection of myocardial dysfunction.

- B. Determining which biomarker correlates with imaging parameters of cardiac remodeling or dysfunction in both phases.

- C. Identifying whether changes in cardiac function immediately after anthracycline administration predict which patients will develop progressive cardiac dysfunction over time.

- D. Evaluating the relationship between marker levels and the patterns of change over time against the primary outcome (evidence of remodeling, decreased EF or CHF).

- E. Exploring disease progression through the longitudinal evaluation of different biomarkers and imaging parameters of cardiac remodeling (measured by changes in LV dimension and wall thickness) and dysfunction (measured by change in EF).

- F. Describing the relationship between early signs of cardiac dysfunction in long-term survivors of childhood cancer to biomarkers of cardiac damage.

- G. Calculating the proportion of patients with evidence of sub-clinical dysfunction (assessed by strain), global dysfunction (EF < 55% or CHF) or remodeling (assessed by LVPWT, TDR) at each time point, and assessing the rate of change in each parameter over time.

The Secondary Outcome Measurement is the Diastolic Function Parameters and their Relationship to Other Parameters at any Study Time Point as Follows

- A. Determining patients with abnormal diastolic function, as indicated by the mitral valve inflow and pulmonary vein profiles and correlate their results with the biomarker and genetic test results.

- B. Determining the relationship among the diastolic function parameters, remodeling parameters and strain parameters.

- C. Determining the relationship among patient demographics (e.g. age at treatment, gender), treatment (e.g. cumulative anthracycline dose, radiation exposure), genetic variants, biomarkers and imaging parameters including diastolic function at baseline and during follow-up in the first and second phases.

If such international short-term and longitudinal investigations are conducted for each type of malignancies, it is believed that rigorous conclusions will be reached regarding successful tailored cardioprotective measures in children treated with anthracycline at risk of cardiotoxicity. The results of such investigation will provide a valuable scientific reference for future research by providing an informative data resource, and a tentative risk prediction model, that will enable thorough understanding of cardiac late effects resulting from anthracyclines in children with cancer and will improve the quality & safety of the care provided to those patients. It is to be

noted that future studies are also needed to identify biomarkers for mitochondria microcirculation and metabolic abnormalities as one mechanism for anthracycline-induced HF and to identify novel biomarkers specific for cardiac remodeling and fibrosis.

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