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Rationale and design of a cohort Study On Primary ovarian insufficiency in female survivors of Hodgkin lymphoma: Influence on long-term Adverse effects (SOPHIA)

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ABSTRACT

Introduction: Hodgkin lymphoma (HL) has become the prototype of a curable disease. However, many young survivors suffer from late adverse effects of treatment. Both chemotherapy (CT) and radiotherapy (RT) may induce primary ovarian insufficiency (POI), which has been associated with reduced bone mineral density (BMD), neurocognitive dysfunction and possibly cardiovascular disease (CVD). While the general assumption is that POI increases CVD risk, other hypotheses postulate reverse causality, suggesting that cardiovascular risk factors determine menopausal age or that biological aging underlies both POI and CVD risk. None of these hypotheses are supported by convincing evidence. Furthermore, most studies on POI-associated conditions have been conducted in women with early natural or surgery-induced menopause with short follow-up times. In this study we will examine the long-term effects of CT- and/or RT-induced POI on BMD, cardiovascular status, neurocognitive function and quality of life (QoL) in female HL survivors.

Methods and analysis: This study will be performed within an existing Dutch cohort of HL survivors. Eligible women were treated for HL at ages 15-39 years in three large hospitals since 1965 and survived for ≥8 years after their diagnosis. Women will be invited to visit a survivorship care outpatient clinic (SSC) for a neurocognitive, cardiovascular and BMD assessment, and asked to complete several questionnaires and to provide a blood sample. Using multivariable regression analyses we will compare the outcomes of HL survivors who developed POI with those who did not. Cardiovascular status will also be compared with women with natural POI.

Ethics and dissemination: This study has been approved by the Institutional Review Board of the Netherlands Cancer Institute and has been registered at "Toetsingonline" from the Dutch Central Committee on Research involving Human Subjects (file number NL44714.031.13). Results will be disseminated through peer-reviewed publications and will be incorporated in follow-up guidelines for HL survivors.

STRENGHTS AND LIMITATIONS

- This study is the first to examine to the long-term effects of chemotherapy- and radiotherapy -induced primary ovarian insufficiency in female Hodgkin lymphoma survivors
- Furthermore, this study is embedded in an infrastructure of several multidisciplinary survivorship care clinics, enabling a broad scope of medical tests and extensive follow-up care

- Results of this study will help to identify and timely refer those Hodgkin lymphoma survivors who are at increased risk for osteoporosis, neurocognitive dysfunction and possibly cardiovascular disease due to treatment-induced primary ovarian insufficiency for interventions in order to reduce morbidity and enhance quality of life
- Moreover, this study sheds light on different hypotheses regarding the association between primary ovarian insufficiency and cardiovascular disease risk
- Data collection is dependent on routine care procedures of the participating survivorship care clinics and of visiting patients

KEY WORDS

Hodgkin lymphoma, cancer survivor, primary ovarian insufficiency, bone mineral density, cardiovascular disease, neurocognitive function

BACKGROUND

Primary ovarian insufficiency in Hodgkin lymphoma survivors

Due to the improvements in treatment since 1960, Hodgkin lymphoma (HL) has become the prototype of a curable malignancy. Nowadays, overall 10-year survival rates exceed 80%.[1-3] However, survivors are faced with several late adverse effects of treatment, such as second malignancies and cardiovascular disease (CVD) [4-10]. Moreover, 30-40% of female HL survivors treated before 1985 developed primary ovarian insufficiency (POI) (menopause before the age of 40 years) [11-15], compared with 1% of women in the general population [16]. Women treated with more recent, less gonadotoxic treatment regimens are likely to have a lower risk, but the long-term risk has not yet been quantified sufficiently.

The risk of POI strongly depends on type of HL treatment, with the highest cumulative risks reported following pelvic RT (81%) and alkylating CT (42-60%).[11-15 17] Older age at treatment (up to 40 years) does not appear to increase risk. Although women treated for HL at older age will develop POI sooner after treatment compared with those treated at younger ages, the cumulative incidence at age 40 is nearly equal in both groups.[11 13 14] In our earlier study among female 5-year HL survivors treated between 1965 and 1995, women developed POI at a median age of 33 years.[11] POI occurring this early potentially has a large impact on quality of life (QoL) as it results in infertility, and menopausal symptoms including hot flushes, vaginal dryness and mood swings that are more severe than after menopause at later ages.[18-20] In addition, POI has been associated with reduced bone mineral density (BMD), and increased risk of CVD and neurocognitive dysfunction.

Bone mineral density

Women who reach menopause early (before age 45 years), have a lower BMD and higher incidence of osteoporosis than women who enter menopause at ages ≥50 years.[21-23] Moreover, an early menopause has been associated with a 1.5-3-fold increased fracture risk.[21 22 24] The most common osteoporotic fractures in postmenopausal women occur in the hip, wrist and spine.[24 25] However, it is unclear whether the association between early menopause and BMD and fracture risk persists over time. Some studies have shown that the association becomes much weaker with increasing age (mainly above age 70) [26-28], while others reported a lifetime increased fracture risk.[22 29] Possibly, not only oestrogens but also aging mechanisms are of importance in BMD status.[30]

So far, many studies have been conducted in women with an early natural or surgery-induced menopause, while studies evaluating the long-term effects of CT- and/or RT-induced POI on BMD and fracture risk are limited. Two small studies among HL survivors reported a significantly reduced BMD after treatment-induced

POI [31 32], while another study found no association.[33] More research is needed to identify the extent of reduced BMD and prevalence of osteoporotic fractures among female HL survivors who developed POI.

Cardiovascular disease

In the general population, early menopause has been associated with an increased incidence of CVD [34-36]. A recent meta-analysis of CVD risk among women with POI showed a pooled HR of 1.6 for total CVD and 1.7 for ischemic heart disease when compared with menopause at ages ≥50 years.[37] Moreover, epidemiological data show a 2% decrease in cardiovascular mortality for each year menopause is delayed.[38] Low levels of testosterone in women have also been associated with increased intima media thickness (IMT) of the carotid artery.[39-41]

An intriguing hypothesis postulates that reverse causality may operate, i.e., that CVD risk factors such as weight, cholesterol and blood pressure determine menopausal age. This is in contrast with the general assumption that endocrine changes due to early menopause are responsible for CVD development. Indeed, in the Framingham Heart Study cohort, a 1% higher premenopausal cardiovascular risk score was associated with a subsequent decrease in menopausal age of 1.8 years.[42] However, it has also been suggested that several risk factors are associated with both CVD and early menopause. A meta-analysis of 22 genome-wide association studies (GWAS) on natural early menopause revealed predominantly genes that are involved in general repair mechanisms [43], arguing for a role of generalized aging rather than ovarian dysfunction in early menopause.

To date, the important question whether accelerated biological aging underlies both early menopause and an increased CVD risk is unresolved and no recent evidence has been provided to support the reverse causality hypothesis. A direct comparison of CVD risk between HL survivors and women with natural POI might provide new insights, as POI among HL survivors is induced by treatment (exogenous factors) instead of natural early depletion of the primordial follicle pool (endogenous factors). If POI would increase CVD risk, this should be considered in the light of an established increased risk of CVD due to mediastinal RT and anthracycline-containing CT.[9]

Neurocognitive function

Although in-vitro studies suggest a neuroprotective effect of oestrogen in the brain, the influence of decreased estrogen levels on cognitive performance is still unclear. Some in-vivo studies have shown an increased risk of neurocognitive impairment or dementia after a surgery-induced early menopause (approximately 1.5-fold), while others found no association.[44-48] These contradicting findings may be due to differences in the

cognitive domains that were evaluated, as not all cognitive functions are equally influenced by oestrogens. Hormonal influences seem to mainly concern aspects of memory, information processing speed and executive functioning.[49 50]

Up to now, the long-term effects of POI on cognition are largely unknown, as most studies had short follow-up times and included only women with menopausal ages above 40 years and/or women who used hormone replacement therapy (HRT). Moreover, the majority of studies looked at the effects after oophorectomy, characterized by an abrupt drop of oestrogen levels, while oestrogen levels may decrease gradually in CT-induced POI occurring many years after treatment.[20 51]

Hormone replacement therapy

Much debate surrounds the use of HRT since the Women's Health Initiative (WHI) study reported increased risks of breast cancer, CVD and cognitive impairment after oestrogen and progestin suppletion.[52] More recent studies suggest that the benefit of oestrogen suppletion strongly depends on starting age and timing with respect to menopause.[53-56] Among HL survivors in the Netherlands, HRT has been mainly prescribed to relieve menopausal symptoms and to prevent osteoporosis.[57] However, in several HL treatment centres, HL survivors have been advised to refrain from using HRT against menopausal symptoms because of a potential increase in breast cancer risk.[58] This provides the unique opportunity to examine the effects of HRT use in this population as the long-term effects of HRT on BMD, CVD and neurocognitive function in HL survivors with POI have not been examined yet.

Aim

This article describes the design and methods of a Study On Primary ovarian insufficiency in female survivors of Hodgkin lymphoma: Influence on long-term Adverse effects (SOPHIA). The study aims to examine the long-term effects of treatment-induced POI on BMD, cardiovascular status, neurocognitive function and QoL. We hypothesize that women with treatment-induced POI will have an increased risk of osteoporosis and neurocognitive dysfunction and a lower QoL than HL survivors without POI. However, based on the hypotheses on reverse causality and biological aging, we hypothesize that CVD risk may not be increased in female HL survivors with POI compared to HL survivors without POI.

Furthermore, we will examine whether long-term effects differ between women with CT- and RT-induced POI, and between acute and more gradually developed POI. We will also investigate the effects of type and timing of HRT on all outcomes if there is sufficient power. Finally, we will compare cardiovascular status and the

possible influence of HRT between HL survivors with treatment-induced POI and women from the general population with natural (non-treatment-induced) POI.

METHODS

Design and study population

Hodgkin lymphoma survivors

The SOPHIA-study is an observational cross-sectional study in which participants will be invited to complete several questionnaires and to visit an outpatient clinic for a neurocognitive, cardiovascular and BMD assessment. Participants will be recruited from a large previously described cohort of 5-year HL survivors treated in the Netherlands between 1965 and 2000 [4 59], which has been extended with more recently treated patients. This study is a collaboration of three large Dutch Medical Centres: The Netherlands Cancer Institute (NKI), VU University Medical Centre (VUmc) and Leiden University Medical Centre (LUMC). Eligible women were treated for HL at the age of 15-39 years at the adult Haematology-Oncology departments of the three medical centres, and survived ≥8 years after HL diagnosis. Exclusion criteria are: current age of ≥75 years, current treatment for a second malignancy, insufficient understanding of the Dutch language or any psychological, familial, sociological or geographical condition that potentially hampers study participation. The study has been approved by the Institutional Review Board of The Netherlands Cancer Institute. Recruitment started in 2014 (VUmc). We will follow the study population longitudinally to examine changes in risk factor and outcomes over time for which additional funding will be acquired.

External control group

To enable the comparison of cardiovascular status between HL survivors and women with natural (non-treatment-induced) POI, we will use data from an ongoing nationwide multicentre study on hypergonadotropic oligomenorrohea/amenorrhea. This study is a collaboration of three University Medical Centres and includes women aged ≥40 years with a diagnosis of polycystic ovarian syndrome (PCOS) or POI between 1992 and 2012. Data collection consists of a cardiovascular risk assessment at the outpatient clinic.[60 61]

Study parameters and data collection

Main outcome measures and other relevant study parameters are briefly described below by method of data collection (see also Table 1). The main exposure POI is defined as amenorrhea for ≥4 months with two serum

follicle-stimulating hormone (FSH) levels in the menopausal range (obtained at least 1 month apart), or amenorrhea for ≥12 months before the age of 40 years.

Table 1. Overview of outcome measures and corresponding data collection methods

Primary exposure and outcomes	Data collection methods		Outcome variables	Justification of methods
Primary ovarian insufficiency	Blood sample	Hormone level	If indicated for routine care: level of follicle-stimulating hormone (FSH) in mIU/mL	Routine care – diagnostic value
	Questionnaire		Date of last menstruation, menopausal age	
	Medical record		Date of last menstruation, menopausal age	
Bone mineral density	Medical tests	DEXA-scan of lumbar spine and hip by means of Hologic Delphi densitometer (VUmc) or General Electric Scanner (LUMC)	BMD values in g/cm^2 T and Z scores Presence of osteopenia (defined as T-scores of -1 to -2.5) Presence of osteoporosis (defined as T-scores of ≥-2.5)	Routine care – diagnostic value The DEXA-scan is most widely used in clinical practice to screen for osteoporosis and regarded as the 'Golden standard'
		Instant vertebral assessment (IVA) by Hologic Delphi densitometer (VUmc) or General Electric Scanner (LUMC)	Vertebral height reduction in % Presence of clinical and non-clinical vertebral fractures	There is a strong additive value of IVA compared with DEXA alone [62-64]
		Anthropomorphic measurements	Height in cm and weight in kg	
	Blood sample	Bone turnover markers	Bone formation by P1NP - mean value in ng/ml Bone resorption by β-CTX - mean value in pg/ml	These markers have been used in previous studies and are recommended for research purposes [65]
		Vitamin D	Level of 25-hydroxyvitamine D in serum in nmol/l	Vitamin D has been associated with bone turnover markers, BMD, fracture risk and risk of falling [66-68]
	Questionnaire	Food frequency questionnaire (FFQ)	Mean score of calcium intake	The FFQ is a validated questionnaire [69] Reference values are available [70]
		General questionnaire	Previous fractures, use of calcium and vitamin D supplements use of glucocorticoids, family history of osteoporosis	
	Medical record		Earlier DEXA-scans (yes,no), if applicable treatment plan for osteoporosis such as vitamin D supplementation, recommendations for lifestyle changes	
Cardiovascular status	Medical tests	Echocardiogram If contra-indicated: cardiac magnetic resonance imaging (MRI)	Abnormalities in heart structure Left ventricular function (LVF) by E/A ratio, deceleration time, isovolumic relaxation time (IVRT), left ventricular ejection fraction (LVEF), diastolic and systolic diameter and volume, E/e' ratio Right ventricular function: tricuspid annular plane systolic excursion (TAPSE) Presence of mitral, aortic or tricuspid valve defects i.e. insufficiencies or stenoses Wall motion score index	Routine care – diagnostic value
		Electrocardiogram (ECG)	Sinus rhythm, QRS complex, ST morphology (elevation or depression), PQ interval and left ventricle hypertrophy	Routine care – diagnostic value
		Coronary computed tomography angiography (CCTA) by a 320-detector	Coronary artery calcium (CAC) score according to Agatston Presence of luminal narrowing and if applicable: type of	High sensitivity and specificity [71] Most valid alternative method for detecting

		row volumetric scanner (Aquilion ONE) (LUMC) and 256 Scanner Philips (VUmc)	narrowing and number of plaques for the left main coronary artery (LMCA), left anterior descending (LAD), circumflex artery (CRX) and right coronary artery (RCA)	significant coronary disease (golden standard is invasive coronary angiography) [72]
		Vascular measurements	Presence of atherosclerosis by carotid intima media thickness (cIMT) and femoral IMT in mm Arterial stiffness (VUmc only)	Predictors of future cardiovascular events [73-75]
		Blood pressure	Mean of three consecutive measurements in mm/Hg	
		Anthropomorphic measurements	Height in cm, weight in kg, body mass index (BMI) in kg/cm^2, hip circumference in cm, waist circumference in cm, hip-waist ratio	
	Blood sample	Biomarkers	Left ventricular function and presence of ischemia and infarction by NT-pro-BNP in pmol/l Chronic inflammation (associated with atherosclerosis) by CRP in mg/L	In general population: strong predictor of coronary heart disease [76 77]
		Lipid spectrum	Total cholesterol, HDL, LDL, triglycerides	Established risk factors for CVD
		Glucose	Fasting blood glucose	Established risk factor for diabetes
		Kidney function	Creatinine, estimated glomerular filtration rate (eGFR)	Routine care before CCTA
	Questionnaire	'General questionnaire'	(Family) history of CVD and risk factors for CVD and if applicable date of diagnosis and treatment	
	Medical record		Cardiovascular risk score based on SCORE chart and Framingham chart, adjusted for age Cardiovascular history, contra-indications for echocardiogram	
Neurocognitive function	Neurocognitive tests	15 Words test	Verbal memory in total number of words	These tests were selected based on their reliability, validity and availability of reference norms. The domains examined are potentially sensitive for the effect of oestrogens [48,62-67]
		Trail making test A&B	Information processing speed) in seconds to complete	
		COWA verbal fluency test	Verbal fluency in total number of words	
		Letter-number sequencing	Working memory in total correct trials	
		WAIS III Digit span	Measures concentration in total number of items/ lists correctly repeated) can be converted to a scaled score, which is an age-based, norm-referenced score for each subject	
		Dutch Adult Reading Test (NART)	Verbal intelligence in mean IQ estimate	
Quality of life	Questionnaire	SF-12	General health	Shortened version of the validated questionnaire SF-36, which has been previously used in Dutch studies [78]
		MOS cognitive functioning scale	Cognitive functioning	Frequently used questionnaire [79]
		Hospital Anxiety and Depression Scale (HADS)	Anxiety and depression	Valid and reliable Dutch reference values are available [80]
		Sexual Activity Questionnaire (SAQ)	Sexual functioning	The SAQ is a valid, reliable and acceptable measure for describing the sexual functioning of women in terms of activity, pleasure and discomfort. It is quick and easy to administer and has good face validity discriminating

				between the sexual functioning of pre- and post-menopausal women [81]
		Shortened fatigue questionnaire (VVV)	Fatigue	Reliable and validated questionnaire [82]

Medical records

Data on HL diagnosis (date, pathology), primary and recurrence treatment (including date, RT fields, chemotherapeutic regimens and doses) and follow-up data have been previously collected from medical records [4]. Since treatment for HL has changed considerably over time, a variety of treatment regimens was used. Primary treatment was usually given according to treatment protocols of the European Organization of Research and Treatment of Cancer (EORTC) and German Hodgkin lymphoma Study Group (GHSG), while treatment for recurrences was generally not standardized. Furthermore, data on reproductive factors (e.g., menopausal age) will be obtained.

Patient questionnaire data

Four questionnaires will be used to ascertain data on women's general characteristics, QoL, calcium intake and menopause related topics. The 'General characteristics questionnaire' will obtain information on the following items: reproductive history (e.g., age at menarche and menopause, parity, hormone use), general cardiovascular history, bone health status (e.g., previous fractures, use of medication), and lifestyle factors (e.g. current and previous smoking habits, alcohol use and physical activity). The 'QoL questionnaire' consists of five short validated and/or frequently used questionnaires regarding health, cognition, sexual activity, depression and fatigue (Table 1). A validated food frequency questionnaire (FFQ) will be used to assess calcium intake [69]. Reference values will be obtained from the report on dietary intake by the Health Council of the Netherlands [70]. The 'Menopause questionnaire' is specifically aimed at postmenopausal women and will collect information on climacteric symptoms (i.e., severity and frequency) and changes in lifestyle factors after the onset of menopause. Data regarding infertility issues will be ascertained for women who experienced POI.

Neurocognitive, cardiovascular and fracture risk assessments

For the neurocognitive assessment, we have chosen tests that measure cognitive domains potentially sensitive for effects of oestrogens.[49 83-88] Tests were selected based on their reliability, validity and availability of Dutch reference norms. The cardiovascular assessment includes an echocardiogram, electrocardiogram (ECG), coronary computed tomography angiography (CCTA), and measurement of the carotid and femoral IMT, blood pressure and hip/waist circumference. The BMD assessment consists of a DEXA-scan with instant vertebral assessment (IVA). These medical tests were chosen based on their availability and use in clinical practice and their evidence-based diagnostic or predictive value (Table 1).

Blood samples

A blood sample will be drawn to examine bone turnover (i.e., β-CTX for bone resorption and P1NP for bone formation) and cardiac (e.g. NT-pro-BNP, CRP, lipid spectrum) markers. Since new techniques in this research field develop rapidly, we will collect an additional blood sample for future analyses on new biomarkers, such as biomarkers predictive for late effects. Moreover, this sample will be used for future DNA extraction and analyses (e.g. to examine modifying effects of genetic factors, such as single nucleotide polymorphisms (SNP's) associated with POI). These blood samples will be frozen and stored at -80 °C.

Study procedures

Recruitment

Women will be recruited through the Survivorship Care outpatient Clinic (SCC) for HL survivors, established by the Dutch nationwide BETER consortium (Better care after Hodgkin lymphoma, Evaluation of long-Term Treatment Effects and screening Recommendations). This consortium consists of haematologists and radiation oncologists of >20 hospitals and has developed evidence-based guidelines on follow-up care, including recommendations on cardiovascular risk assessment in order to reduce morbidity and mortality [89]. The three medical centres participating in the SOPHIA-study all have an active SCC where HL survivors can be screened. Approximately 30% of the 5-year HL survivors already receive routine follow-up care on a yearly basis in their original HL treatment centre. All 5-year HL survivors who are currently not under surveillance will be invited for screening (if treated at the age of 15-60 years and currently aged <75 years) by the BETER consortium in the upcoming years.

If a woman, eligible for the SOPHIA-study, visits the SCC of NKI, LUMC or VUmc (either during an intake or follow-up care visit), she will be invited by her treating physician. As mentioned above, follow-up care is provided according to the BETER guidelines and depends on the specific treatments a patient received (e.g., chest or pelvic RT, anthracycline-containing CT). As some of the medical tests in the SOPHIA-study are incorporated in the BETER guidelines, these tests may be part of routine care. Therefore, the physician will determine for each patient which medical tests will be performed for routine care and which will be additionally performed for research purposes. Tests that are considered for routine care may have been recently performed in a participant. If there is no clinical reason to repeat the test, the result of the previous test will be abstracted from the medical record. More details on the distinction between routine care and research tests, and the study procedures are described in Figure 1.

We aim to integrate study participation as much as possible with the routine care provided at the SCC. Whether or not a patient is willing to participate in the study will not have any influence on the routine care she receives during follow-up.

Study implementation

If the patient is interested to participate in the SOPHIA-study, the treating physician will hand out an invitation for the SOPHIA-study, together with a patient information letter and an informed consent form. After one or two weeks, the treating physician or research nurse will contact the patient by telephone to answer any remaining questions. If the woman agrees to participate, she will be asked to return the signed informed consent form, and subsequently the 'General questionnaire' and 'Menopause questionnaire' (if applicable) will be sent to her home. Patients will be asked to bring their completed questionnaires with them to a follow-visit at the SSC or to return the questionnaires by mail.

The neurocognitive, cardiovascular and BMD assessments will be performed during a follow-up visit at the SCC. Ideally, all medical tests will be performed during two to three follow-up visits, depending on availability and timing of other routine medical care tests (e.g. breast cancer screening). The planned tests with allocated time are shown in Figure 1.

Patients will be tested for renal failure before undergoing the CCTA. Women with severe renal insufficiency, defined as a e-GFR value of <60 ml per minute per 1.73m^2, will undergo a computed tomography coronary calcium score without contrast fluid.

Blood will be drawn at two time points. The first blood sample will be taken at the SCC during a routine care blood withdrawal. The second blood sample will be drawn in fasting state before the CCTA. Patients are offered the possibility to perform the neurocognitive tests at home. In that case, a separate appointment will be made. To ensure sufficient time between two memory tests, patients will be asked to complete the FFQ during this appointment.

Statistical issues

Power calculation

Approximately 500 women are eligible for participation in the three selected hospitals. Based on our previous studies we expect that 60% of the eligible women will participate in the current study (N=300). Power calculations were performed separately for the outcomes BMD, cardiovascular status, neurocognitive function

and QoL. When comparing BMD of women who developed POI (expected N=60 (20%)) with those who did not (expected N=240), there is over 80% power to detect a difference of 0.05 g/cm2 in BMD (1.00 (SD 0.1) vs 1.05 (SD 0.1)).

The power calculation for cardiovascular status is based on the IMT measurement. There is over 80% power to detect a difference of 0.1 mm in mean IMT between women with POI and women with normal menopausal ages (0.6 (SD 0.2) versus 0.5 (SD 0.2). An increase of 0.1 mm in IMT has been associated with an increase in risk of 12% for myocardial infarction.[74]

Previous retrospective neuropsychological studies, in which effects of systemic cancer treatments and POI on cognitive functioning were examined, have yielded significant findings with somewhat smaller group sizes of 39 and 53 patients.[90-92]

For the outcome QoL, we followed the calculations of Cohen.[93] The proposed study has over 80% power to detect moderate difference between women who developed POI and those who did not. All power calculations used a 5% chance for a Type-1 error and a minimal effect size of 0.5.

Statistical analyses

Outcomes of female HL survivors who developed POI will be compared with those of female HL survivors who did not by using chi square tests or Fisher's exact tests (categorical variables) and two tailed t-tests (continuous variables) after appropriate transformation, if necessary. Multivariate regression analyses will be used to examine the effects of POI, menopausal age and HRT use on the primary outcome variables (BMD, cardiovascular status and neurocognitive function) and to assess the effect of these primary outcome variables on QoL. All analyses will be adjusted for confounders (HL treatment regimen, lifestyle factors, reproductive factors, climacteric symptoms, medication) where applicable. Effect modification and mediation will be tested using interaction terms. We will also perform subgroup analyses to evaluate the difference between a CT- and RT-induced POI in more detail and we will compare cardiovascular status between HL survivors with POI and other women with natural POI. P values <0.05 will be considered statistically significant.

DISCUSSION

In the current study, the long-term effects of CT- and/or RT-induced POI on BMD, cardiovascular status, neurocognitive function and QoL will be examined by measurements within a cohort of female HL survivors. Approximately 30-40% of the female survivors in our HL cohort treated between 1965 and 1985 experienced

treatment-induced POI. The majority of these women have now been postmenopausal for over 20 to 30 years which enables us to examine the very late effects of POI.

Treatment-induced POI might put female HL survivors at high risk for developing adverse POI-associated conditions such as osteoporosis, neurocognitive dysfunction and CVD, while they already have an increased risk for late adverse effects due to the HL treatment itself.[4 9 21 37 46] This may lead to problems in daily functioning and can have a large impact on their QoL, in particular because the conditions may occur at very young ages. Results of this study will help to identify those HL survivors who are at increased risk for osteoporosis, neurocognitive dysfunction and possibly CVD due to treatment-induced POI. This enables timely referral of high-risk women for interventions, thereby reducing (subclinical) adverse events and improving QoL. Moreover, by identifying these long-term risks, physicians can better inform women with POI in the future. Findings of this study will also be relevant for other female cancer patients who received gonadotoxic treatment at premenopausal ages. CT is a major contributor to the development of POI and its use has intensified considerably over the years in many malignancies.[94 95] Therefore, it is expected that the occurrence of POI-associated adverse effects will increase in female cancer survivors in the near future. Since HRT has become subject of much debate in recent years, investigating the effects of HRT on POI-associated conditions will produce valuable knowledge with regard to the HRT-suppletion policy for female cancer survivors in the Netherlands.

Finally, this study provides a unique possibility to challenge the conventional view that reproductive hormone deprivation in females is of key importance in CVD development. This is relevant in the light of other hypotheses that general biological aging mechanisms underlie a combination of POI and CVD, or that CVD risk factors determine age at menopause (reverse causality hypothesis).[42] Comparison of cardiovascular status between women with POI after HL treatment and women with natural POI will allow examination of a cause-effect relationship between early menopause and CVD. We will be able to adjust for the potential effects of HL treatment on CVD risk, as extensive data on HL treatment regimens are available within our cohort.

This study has several notable strengths and limitations. First, this study is embedded in an infrastructure of several multidisciplinary SCCs, enabling a broad scope of medical tests and extensive follow-up care. Detailed treatment and reproductive data will be available from medical records and patient questionnaires. Second, women received a variety of treatments and have long-term follow-up, rendering it possible to examine the effects of different HL treatments, POI and menopausal age on several outcomes. A limitation of the study includes patient selection. Due to the high risk of late adverse effects in HL survivors, some women are already

deceased or not able to participate in the SOPHIA-study. Moreover, some women are under surveillance in a local hospital rather than their original HL treatment centre, while women who are (feeling) healthy may not visit the SCC because of medical costs and/or other obligations (e.g., work, family). We will account for this by obtaining medical data for women under surveillance in others hospitals, and we have near complete data on important competing late adverse effects such as CVD, second malignancies and vital status, as this study is nested within an existing cohort.

Another limitation is that this study is dependent on routine care procedures of the participating SSCs. Therefore, differences between SSCs may occur regarding available equipment and registration of results from medical tests. In addition, the time interval between two medical tests may be variable between patients due to planning issues (e.g., long waiting lists, the aim to plan multiple medical tests in one day), or because one medical test has already been performed in the past year and will not be repeated. We made a standardized abstraction form to ensure all relevant data are gathered and differences between timing of medical tests and hospitals will be accounted for in the analyses.

In conclusion, this article describes the study protocol of the SOPHIA-study which aims to increase knowledge about BMD, cardiovascular status and neurocognitive function in long-term female HL survivors with and without treatment-induced POI, and the potential influence of these long-term effects on QoL. Results of this study will lead to the identification of those HL survivors who are at increased risk for osteoporosis, neurocognitive dysfunction and possibly CVD due to treatment-induced POI. This enables timely referral of high-risk women for interventions, thereby reducing (subclinical) adverse events and improving QoL. Furthermore, results will shed light on existing hypotheses regarding the association between POI and CVD risk. Moreover, HL survivors or other cancer survivors who will experience treatment-induced POI in the future can be better informed about potential long-term effects.

AUTHORS' CONTRIBUTIONS

The study protocol has been written by IK, AOvW, BA, EvD-dB and FvL. All authors contributed to study design. JZ, LD, and BA are responsible for patient accrual and inclusion, and JZ, YA, SB, LM, ES, PL, and LD and BA are responsible for the assessment of the outcome variables.

FvL is the principal investigator and responsible for the funding of the study.

All authors revised the manuscript critically for intellectual content and have approved the final manuscript.

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COMPETING INTERESTS

JZ declares she has conducted a research project funded by Roche in the past two years (unrelated to the current study). CBL's department of Reproductive Medicine has received educational and research grants from Merck Serono, Ferring and Auxogyn, and he received speakers fees from MSD, Merck Serono, Ferring and Auxogyn. He is also a consultant for Ferring. PL provided advice to Friesland Campina. All other authors declare no competing interests.

ETHICS AND DISSEMINATION

This study has been approved by the Institutional Review Board of the Netherlands Cancer Institute and has been registered at "Toetsingonline" from the Dutch Central Committee on Research involving Human Subjects (file number NL44714.031.13). Reporting of serious adverse events was exempted for this study, as the burden and risks associated with participation are very low and in accordance with routine medical care. Results will be disseminated through peer-reviewed publications and will be incorporated in follow-up guidelines for HL survivors.

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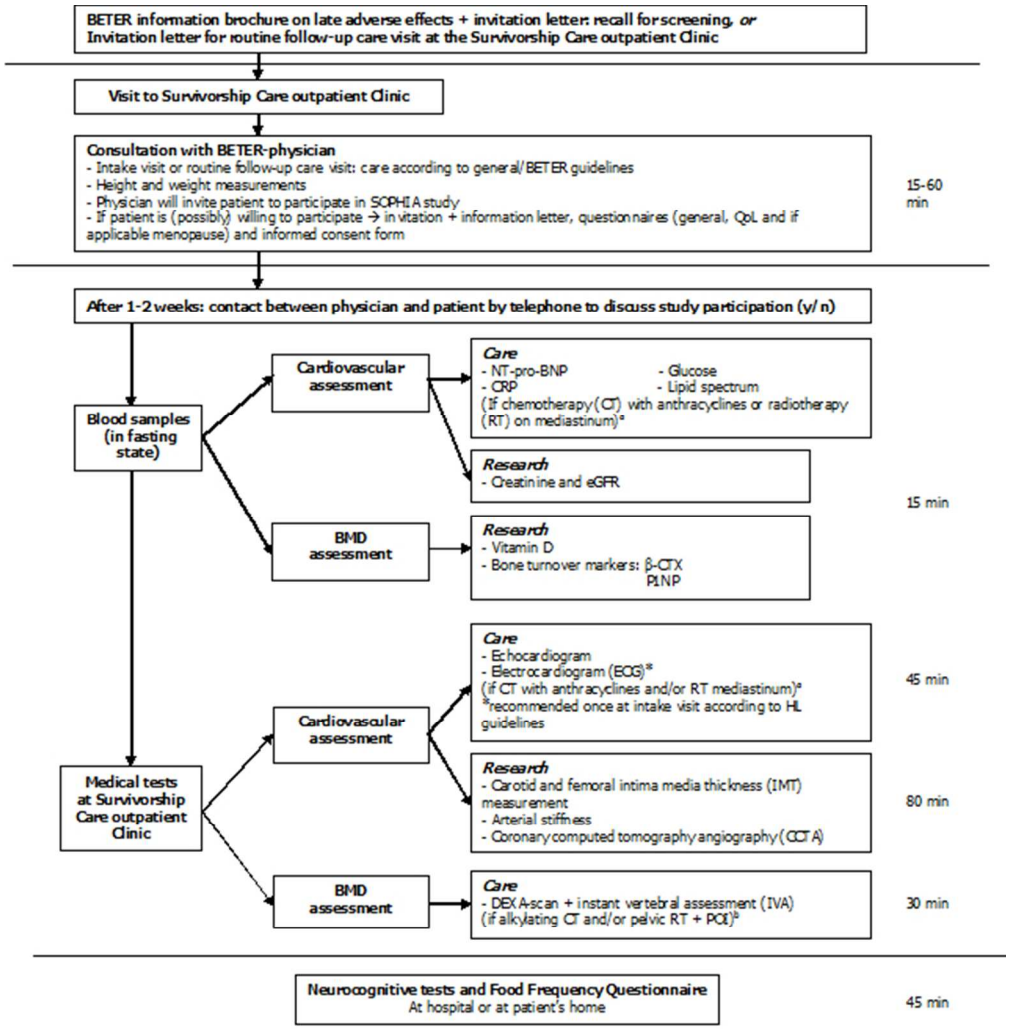
FIGURE LEGENDS

Figure 1. Study procedures and patient burden, stratified by medical tests for routine care and research

Abbreviations: RT = radiotherapy; CT = chemotherapy; BETTER = Better care after Hodgkin lymphoma, Evaluation of long-Term Treatment Effects and screening Recommendations; CRP = C-reactive protein; BMD = bone mineral density; DEXA = Dual Energy X-ray Absorptiometry

[a] Expected for > 90% of women [b] Expected for 15-40% of women

In case criteria for care are not fulfilled, tests will be performed for research purpose



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169x172mm (96 x 96 DPI)

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Rationale and design of a cohort Study On Primary ovarian insufficiency in female survivors of Hodgkin lymphoma: Influence on long-term Adverse effects (SOPHIA)

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Rationale and design of a cohort Study On Primary ovarian insufficiency in female survivors of Hodgkin lymphoma: Influence on long-term Adverse effects (SOPHIA)

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ABSTRACT

Introduction: Hodgkin lymphoma (HL) has become the prototype of a curable disease. However, many young survivors suffer from late adverse effects of treatment. Both chemotherapy (CT) and radiotherapy (RT) may induce primary ovarian insufficiency (POI), which has been associated with reduced bone mineral density (BMD), neurocognitive dysfunction and possibly cardiovascular disease (CVD). While the general assumption is that POI increases CVD risk, other hypotheses postulate reverse causality, suggesting that cardiovascular risk factors determine menopausal age or that biological aging underlies both POI and CVD risk. None of these hypotheses are supported by convincing evidence. Furthermore, most studies on POI-associated conditions have been conducted in women with early natural or surgery-induced menopause with short follow-up times. In this study we will examine the long-term effects of CT- and/or RT-induced POI on BMD, cardiovascular status, neurocognitive function and quality of life (QoL) in female HL survivors.

Methods and analysis: This study will be performed within an existing Dutch cohort of HL survivors. Eligible women were treated for HL at ages 15-39 years in three large hospitals since 1965 and survived for ≥8 years after their diagnosis. Women visiting a survivorship care outpatient clinic (SSC) will be invited for a neurocognitive, cardiovascular and BMD assessment, and asked to complete several questionnaires and to provide a blood sample. Using multivariable regression analyses we will compare the outcomes of HL survivors who developed POI with those who did not. Cardiovascular status will also be compared with women with natural POI.

Ethics and dissemination: This study has been approved by the Institutional Review Board of the Netherlands Cancer Institute and has been registered at "Toetsingonline" from the Dutch Central Committee on Research involving Human Subjects (file number NL44714.031.13). Results will be disseminated through peer-reviewed publications and will be incorporated in follow-up guidelines for HL survivors.

STRENGHTS AND LIMITATIONS

- This study is the first to examine to the long-term effects of chemotherapy- and radiotherapy -induced primary ovarian insufficiency in female Hodgkin lymphoma survivors
- Furthermore, this study is embedded in an infrastructure of several multidisciplinary survivorship care clinics, enabling a broad scope of medical tests and extensive follow-up care

- Results of this study may help to identify and timely refer those Hodgkin lymphoma survivors who are at increased risk for osteoporosis, neurocognitive dysfunction and possibly cardiovascular disease due to treatment-induced primary ovarian insufficiency for interventions in order to reduce morbidity and enhance quality of life
- Moreover, this study sheds light on different hypotheses regarding the association between primary ovarian insufficiency and cardiovascular disease risk
- Data collection is dependent on routine care procedures of the participating survivorship care clinics and of visiting patients

KEY WORDS

Hodgkin lymphoma, cancer survivor, primary ovarian insufficiency, bone mineral density, cardiovascular disease, neurocognitive function

BACKGROUND

Primary ovarian insufficiency in Hodgkin lymphoma survivors

Due to the improvements in treatment since 1960, Hodgkin lymphoma (HL) has become the prototype of a curable malignancy. Nowadays, overall 10-year survival rates exceed 80%.[1-3] However, survivors are faced with several late adverse effects of treatment, such as second malignancies and cardiovascular disease (CVD) [4-10]. Moreover**,** 30-40% of female HL survivors treated before 1985 developed primary ovarian insufficiency (POI) (menopause before the age of 40 years) [11-15], compared with 1% of women in the general population [16]. Women treated with more recent, less gonadotoxic treatment regimens are likely to have a lower risk, but the long-term risk has not yet been quantified sufficiently.

The risk of POI strongly depends on type of HL treatment, with the highest cumulative risks reported following pelvic RT (81%) and alkylating CT (42-60%).[11-15 17] Older age at treatment (up to 40 years) does not appear to increase risk. Although women treated for HL at older age will develop POI sooner after treatment compared with those treated at younger ages, the cumulative incidence at age 40 is nearly equal in both groups.[11 13 14] In our earlier study among female 5-year HL survivors treated between 1965 and 1995, women developed POI at a median age of 33 years (range 19-39 years).[11] POI occurring this early potentially has a large impact on quality of life (QoL) as it results in infertility, and menopausal symptoms including hot flushes, vaginal dryness and mood swings that are more severe than after menopause at later ages.[18-20] In addition, POI has been associated with reduced bone mineral density (BMD), and increased risk of CVD and neurocognitive dysfunction.

Bone mineral density

Women who reach menopause early (before age 45 years), have a lower BMD and higher incidence of osteoporosis than women who enter menopause at ages ≥50 years.[21-23] Moreover, an early menopause has been associated with a 1.5-3-fold increased fracture risk.[21 22 24] The most common osteoporotic fractures in postmenopausal women occur in the hip, wrist and spine.[24 25] However, it is unclear whether the association between early menopause and BMD and fracture risk persists over time. Some studies have shown that the association becomes much weaker with increasing age (mainly above age 70) [26-28], while others reported a lifetime increased fracture risk.[22 29] Possibly, not only oestrogens but also aging mechanisms are of importance in BMD status.[30]

So far, many studies have been conducted among breast cancer survivors or in women with an early natural or surgery-induced menopause, while studies evaluating the long-term effects of CT- and/or RT-induced POI on

BMD and fracture risk are limited. Two small studies among HL survivors reported a significantly reduced BMD after treatment-induced POI [31 32], while another study found no association.[33] Since HL survivors develop POI at a younger age than breast cancer survivors or the general population, more research is needed to identify the extent of reduced BMD and prevalence of osteoporotic fractures among female HL survivors who developed POI.

Cardiovascular disease

In the general population, early menopause has been associated with an increased incidence of CVD [34-36]. A recent meta-analysis of CVD risk among women with POI showed a pooled HR of 1.6 for total CVD and 1.7 for ischemic heart disease when compared with menopause at ages ≥50 years.[37] Moreover, epidemiological data show a 2% decrease in cardiovascular mortality for each year menopause is delayed.[38] Low levels of testosterone in women have also been associated with increased intima media thickness (IMT) of the carotid artery.[39-41]

An intriguing hypothesis postulates that reverse causality may operate, i.e., that CVD risk factors such as weight, cholesterol and blood pressure determine menopausal age. This is in contrast with the general assumption that endocrine changes due to early menopause are responsible for CVD development. Indeed, in the Framingham Heart Study cohort, a 1% higher premenopausal cardiovascular risk score was associated with a subsequent decrease in menopausal age of 1.8 years.[42] However, it has also been suggested that several risk factors are associated with both CVD and early menopause. A meta-analysis of 22 genome-wide association studies (GWAS) on natural early menopause revealed predominantly genes that are involved in general repair mechanisms [43], arguing for a role of generalized aging rather than ovarian dysfunction in early menopause.

To date, the important question whether accelerated biological aging underlies both early menopause and an increased CVD risk is unresolved and no recent evidence has been provided to support the reverse causality hypothesis. A direct comparison of CVD risk between HL survivors and women with natural POI might provide new insights, as POI among HL survivors is induced by treatment (exogenous factors) instead of natural early depletion of the primordial follicle pool (endogenous factors). If POI would increase CVD risk, this should be considered in the light of an established increased risk of CVD due to mediastinal RT and anthracycline-containing CT.[9]

Neurocognitive function

Although in-vitro studies suggest a neuroprotective effect of oestrogen in the brain, the influence of decreased estrogen levels on cognitive performance is still unclear. Some in-vivo studies have shown an increased risk of neurocognitive impairment or dementia after a surgery-induced early menopause (approximately 1.5-fold), while others found no association.[44-48] These contradicting findings may be due to differences in the cognitive domains that were evaluated, as not all cognitive functions are equally influenced by oestrogens. Hormonal influences seem to mainly concern aspects of memory, information processing speed and executive functioning.[49 50]

Up to now, the long-term effects of POI on cognition are largely unknown, as most studies had short follow-up times and included only women with menopausal ages above 40 years and/or women who used hormone replacement therapy (HRT). Moreover, the majority of studies looked at the effects after oophorectomy, characterized by an abrupt drop of oestrogen levels, while oestrogen levels may decrease gradually in CT-induced POI occurring many years after treatment.[20 51] Preliminary data on POI in HL survivors within our cohort show that women who developed POI had a median duration of ovarian function after HL treatment of 4 years (interquartile range 1-10 years).

Hormone replacement therapy

Much debate surrounds the use of HRT since the Women's Health Initiative (WHI) study reported increased risks of breast cancer, CVD and cognitive impairment after oestrogen and progestin suppletion.[52] More recent studies suggest that the benefit of oestrogen suppletion strongly depends on starting age and timing with respect to menopause.[53-56] Among HL survivors in the Netherlands, HRT has been mainly prescribed to relieve menopausal symptoms and to prevent osteoporosis.[57] However, in several HL treatment centres, HL survivors have been advised to refrain from using HRT against menopausal symptoms because of a potential increase in breast cancer risk.[58] This provides the unique opportunity to examine the effects of HRT use in this population as the long-term effects of HRT on BMD, CVD and neurocognitive function in HL survivors with POI have not been examined yet.

Aim

This article describes the design and methods of a Study On Primary ovarian insufficiency in female survivors of Hodgkin lymphoma: Influence on long-term Adverse effects (SOPHIA). The primary aim of this study is to examine the long-term effects of treatment-induced POI on BMD, cardiovascular status, neurocognitive

function and QoL. We hypothesize that women with treatment-induced POI will have an increased risk of osteoporosis and neurocognitive dysfunction and a lower QoL than HL survivors without POI. However, based on the hypotheses on reverse causality and biological aging, we hypothesize that CVD risk may not be increased in female HL survivors with POI compared to HL survivors without POI.

The secondary aims of this study are:

1. To examine whether long-term effects differ between women with CT- and RT-induced POI
2. To examine whether long-term effects differ between acute (<1 year after HL treatment) and more gradually (≥ 1 year after HL treatment) developed POI if there is sufficient power.
3. To investigate the effects of type and timing of HRT on all outcomes if there is sufficient power.
4. To compare cardiovascular status and the possible influence of HRT between HL survivors with treatment-induced POI and women from the general population with natural (non-treatment-induced) POI.

METHODS

Design and study population

Hodgkin lymphoma survivors

The SOPHIA-study is an observational cross-sectional study among female HL survivors who are being followed in an outpatient survivorship care clinic. Participants will be invited for a neurocognitive, cardiovascular and BMD assessment and asked to complete several questionnaires and to provide a blood sample. Participants will be recruited from a large previously described cohort of 5-year HL survivors treated in the Netherlands between 1965 and 2000 [4 59], which has been extended with more recently treated patients. Registry data on HL patients treated before 1965 are not available.

This study is a collaboration of three large Dutch Medical Centres: The Netherlands Cancer Institute (NKI), VU University Medical Centre (VUmc) and Leiden University Medical Centre (LUMC). Eligible women were treated for HL at the age of 15-39 years at the adult Haematology-Oncology departments of the three medical centres,. and survived ≥8 years after HL diagnosis. The latter criterion was chosen because we are interested in the long-term effects of POI. Exclusion criteria are: current age of ≥75 years, current treatment for a second malignancy, insufficient understanding of the Dutch language or any psychological, familial, sociological or geographical condition that potentially hampers study participation. General patient characteristics, HL treatment data and follow-up data on vital status, second malignancies and CVD are already available for all 8-year HL-survivors in these three hospitals, enabling us to monitor possible differences between patients who participate and those

who decline. Also, we will be able to examine whether eligible 8-year survivors who died before study invitation died due to one of our outcomes of interest. Due to the high risk of late adverse effects in HL survivors, some women are already deceased. If it would turn out that a relatively large proportion of patients in the POI group (compared to the comparison group) has died of CVD, we will be able to report this, which is a big advantage in a cross-sectional study.

The study has been approved by the Institutional Review Board of The Netherlands Cancer Institute Recruitment started in 2014 (VUmc). We will follow the study population longitudinally to examine changes in risk factor and outcomes over time for which additional funding will be acquired. Moreover, eligible women will be followed through clinical care, where permission is asked to store future blood samples as well.

External control group

To enable the comparison of cardiovascular status between HL survivors and women with natural (non-treatment-induced) POI, we will use data from an ongoing nationwide multicentre study on hypergonadotropic oligomenorrohea/amenorrhea. This study is a collaboration of three University Medical Centres and includes women aged ≥40 years with a diagnosis of polycystic ovarian syndrome (PCOS) or POI between 1992 and 2012. Data collection consists of a cardiovascular risk assessment at the outpatient clinic.[60 61]

Study parameters and data collection

Main outcome measures and other relevant study parameters are briefly described below by method of data collection (see also Table 1). The main exposure POI is defined as amenorrhea for ≥4 months with two serum follicle-stimulating hormone (FSH) levels in the menopausal range (obtained at least 1 month apart), or amenorrhea for ≥12 months before the age of 40 years. In case a woman has already been postmenopausal for many years at study enrolment, POI is defined as the date of or age at last menstruation. Because we performed earlier studies on POI, for the majority of women we already know their menopausal status and age, either from the medical records or from questionnaires sent in the 1990s-2000s. For the remaining women these data will be abstracted from the medical records and/or obtained through the patient questionnaire.

Table 1. Overview of outcome measures and corresponding data collection methods

Primary exposure and outcomes	Data collection methods		Outcome variables	Justification of methods
Primary ovarian insufficiency	Blood sample	Hormone level	If indicated for routine care: level of follicle-stimulating hormone (FSH) in mIU/mL	Routine care – diagnostic value
	Questionnaire		Date of last menstruation, menopausal age	
	Medical record		Date of last menstruation, menopausal age	
Bone mineral density	Medical tests	DEXA-scan of lumbar spine and hip by means of Hologic Delphi densitometer (VUmc) or General Electric Scanner (LUMC)	BMD values in g/cm^2 T and Z scores Presence of osteopenia (defined as T-scores of -1 to -2.5) Presence of osteoporosis (defined as T-scores of ≥-2.5)	Routine care – diagnostic value The DEXA-scan is most widely used in clinical practice to screen for osteoporosis and regarded as the 'Golden standard'
		Instant vertebral assessment (IVA) by Hologic Delphi densitometer (VUmc) or General Electric Scanner (LUMC)	Vertebral height reduction in % Presence of clinical and non-clinical vertebral fractures	There is a strong additive value of IVA compared with DEXA alone [62-64]
		Anthropomorphic measurements	Height in cm and weight in kg	
	Blood sample	Bone turnover markers	Bone formation by P1NP - mean value in ng/ml Bone resorption by β-CTX - mean value in pg/ml	These markers have been used in previous studies and are recommended for research purposes [65]
		Vitamin D	Level of 25-hydroxyvitamine D in serum in nmol/l	Vitamin D has been associated with bone turnover markers, BMD, fracture risk and risk of falling [66-68]
	Questionnaire	Food frequency questionnaire (FFQ)	Mean score of calcium intake	The FFQ is a validated questionnaire [69] Reference values are available [70]
		General questionnaire	Previous fractures, use of calcium and vitamin D supplements use of glucocorticoids, family history of osteoporosis	
	Medical record		Earlier DEXA-scans (yes,no), if applicable treatment plan for osteoporosis such as vitamin D supplementation, recommendations for lifestyle changes	
Cardiovascular status	Medical tests	Echocardiogram If contra-indicated: cardiac magnetic resonance imaging (MRI)	Abnormalities in heart structure Left ventricular function (LVF) by E/A ratio, deceleration time, isovolumic relaxation time (IVRT), left ventricular ejection fraction (LVEF), diastolic and systolic diameter and volume, E/e' ratio Right ventricular function: tricuspid annular plane systolic excursion (TAPSE) Presence of mitral, aortic or tricuspid valve defects i.e. insufficiencies or stenoses Wall motion score index	Routine care – diagnostic value
		Electrocardiogram (ECG)	Sinus rhythm, QRS complex, ST morphology (elevation or depression), PQ interval and left ventricle hypertrophy	Routine care – diagnostic value
		Coronary computed tomography angiography (CCTA) by a 320-detector	Coronary artery calcium (CAC) score according to Agatston Presence of luminal narrowing and if applicable: type of	High sensitivity and specificity [71] Most valid alternative method for detecting

		row volumetric scanner (Aquilion ONE) (LUMC) and 256 Scanner Philips (VUmc)	narrowing and number of plaques for the left main coronary artery (LMCA), left anterior descending (LAD), circumflex artery (CRX) and right coronary artery (RCA)	significant coronary disease (golden standard is invasive coronary angiography) [72]
		Vascular measurements	Presence of atherosclerosis by carotid intima media thickness (cIMT) and femoral IMT in mm Arterial stiffness (VUmc only)	Predictors of future cardiovascular events [73-75]
		Blood pressure	Mean of three consecutive measurements in mm/Hg	
		Anthropomorphic measurements	Height in cm, weight in kg, body mass index (BMI) in kg/cm^2, hip circumference in cm, waist circumference in cm, hip-waist ratio	
	Blood sample	Biomarkers	Left ventricular function and presence of ischemia and infarction by NT-pro-BNP in pmol/l Chronic inflammation (associated with atherosclerosis) by CRP in mg/L	In general population: strong predictor of coronary heart disease [76 77]
		Lipid spectrum	Total cholesterol, HDL, LDL, triglycerides	Established risk factors for CVD
		Glucose	Fasting blood glucose	Established risk factor for diabetes
		Kidney function	Creatinine, estimated glomerular filtration rate (eGFR)	Routine care before CCTA
	Questionnaire	'General questionnaire'	(Family) history of CVD and risk factors for CVD and if applicable date of diagnosis and treatment	
	Medical record		Cardiovascular risk score based on SCORE chart and Framingham chart, adjusted for age Cardiovascular history, contra-indications for echocardiogram	
Neurocognitive function	Neurocognitive tests	15 Words test	Verbal memory in total number of words	These tests were selected based on their reliability, validity and availability of reference norms. The domains examined are potentially sensitive for the effect of oestrogens [48,62-67]
		Trail making test A&B	Information processing speed) in seconds to complete	
		COWA verbal fluency test	Verbal fluency in total number of words	
		Letter-number sequencing	Working memory in total correct trials	
		WAIS III Digit span	Measures concentration in total number of items/ lists correctly repeated) can be converted to a scaled score, which is an age-based, norm-referenced score for each subject	
		Dutch Adult Reading Test (NART)	Verbal intelligence in mean IQ estimate	
Quality of life	Questionnaire	SF-12	General health	Shortened version of the validated questionnaire SF-36, which has been previously used in Dutch studies [78]
		MOS cognitive functioning scale	Cognitive functioning	Frequently used questionnaire [79]
		Hospital Anxiety and Depression Scale (HADS)	Anxiety and depression	Valid and reliable Dutch reference values are available [80]
		Sexual Activity Questionnaire (SAQ)	Sexual functioning	The SAQ is a valid, reliable and acceptable measure for describing the sexual functioning of women in terms of activity, pleasure and discomfort. It is quick and easy to administer and has good face validity discriminating

				between the sexual functioning of pre- and post-menopausal women [81]
		Shortened fatigue questionnaire (VVV)	Fatigue	Reliable and validated questionnaire [82]

Medical records

Data on HL diagnosis (date, pathology), primary and recurrence treatment (including date, RT fields, chemotherapeutic regimens and doses) and follow-up data have been previously collected from medical records [4]. Since treatment for HL has changed considerably over time, a variety of treatment regimens was used. Primary treatment was usually given according to treatment protocols of the European Organization of Research and Treatment of Cancer (EORTC) and German Hodgkin lymphoma Study Group (GHSG), while treatment for recurrences was generally not standardized. Furthermore, data on reproductive factors (e.g., menopausal age) will be obtained.

Patient questionnaire data

Four questionnaires will be used to ascertain data on women's general characteristics, QoL, calcium intake and menopause related topics. The 'General characteristics questionnaire' will obtain information on the following items: reproductive history (e.g., age at menarche and menopause, parity, hormone use), general cardiovascular history, bone health status (e.g., previous fractures, use of medication), and lifestyle factors (e.g. current and previous smoking habits, alcohol use and physical activity). The 'QoL questionnaire' consists of five short validated and/or frequently used questionnaires regarding health, cognition, sexual activity, depression and fatigue (Table 1). A validated food frequency questionnaire (FFQ) will be used to assess calcium intake [69]. Reference values will be obtained from the report on dietary intake by the Health Council of the Netherlands [70]. The 'Menopause questionnaire' is specifically aimed at postmenopausal women and will collect information on climacteric symptoms (i.e., severity and frequency) and changes in lifestyle factors after the onset of menopause. Data regarding infertility issues will be ascertained for women who experienced POI.

Neurocognitive, cardiovascular and fracture risk assessments

For the neurocognitive assessment, we have chosen tests that measure cognitive domains potentially sensitive for effects of oestrogens.[49 83-88] Tests were selected based on their reliability, validity and availability of Dutch reference norms. The cardiovascular assessment includes an echocardiogram, electrocardiogram (ECG), coronary computed tomography angiography (CCTA), and measurement of the carotid and femoral IMT, blood pressure and hip/waist circumference. The BMD assessment consists of a DEXA-scan with instant vertebral assessment (IVA). These medical tests were chosen based on their availability and use in clinical practice and their evidence-based diagnostic or predictive value (Table 1).

Blood samples

A blood sample will be drawn to examine bone turnover (i.e., β-CTX for bone resorption and P1NP for bone formation) and cardiac (e.g. NT-pro-BNP, CRP, lipid spectrum) markers. Since new techniques in this research field develop rapidly, we will collect an additional blood sample for future analyses on new biomarkers, such as biomarkers predictive for late effects. Moreover, this sample will be used for future DNA extraction and analyses (e.g. to examine modifying effects of genetic factors, such as single nucleotide polymorphisms (SNP's) associated with POI). These blood samples will be frozen and stored at -80 °C.

Study procedures

Recruitment

Women will be recruited through the Survivorship Care outpatient Clinic (SCC) for HL survivors, established by the Dutch nationwide BETER consortium (Better care after Hodgkin lymphoma, Evaluation of long-Term Treatment Effects and screening Recommendations). This consortium consists of haematologists and radiation oncologists of >20 hospitals and has developed evidence-based guidelines on follow-up care, including recommendations on cardiovascular risk assessment in order to reduce morbidity and mortality [89]. The three medical centres participating in the SOPHIA-study all have an active SCC where HL survivors can be screened. Approximately 30% of the 5-year HL survivors already receive routine follow-up care on a yearly basis in their original HL treatment centre. All 5-year HL survivors who are currently not under surveillance will be invited for screening (if treated at the age of 15-60 years and currently aged <75 years) by the BETER consortium in the upcoming years.

If a woman, eligible for the SOPHIA-study, visits the SCC of NKI, LUMC or VUmc (either during an intake or follow-up care visit), she will be invited by her treating physician. As mentioned above, follow-up care is provided according to the BETER guidelines and depends on the specific treatments a patient received (e.g., chest or pelvic RT, anthracycline-containing CT). As some of the medical tests in the SOPHIA-study are incorporated in the BETER guidelines, these tests may be part of routine care. Therefore, the physician will determine for each patient which medical tests will be performed for routine care and which will be additionally performed for research purposes. Tests that are considered for routine care may have been recently performed in a participant. If there is no clinical reason to repeat the test, the result of the previous test will be abstracted from the medical record. More details on the distinction between routine care and research tests, and the study procedures are described in Figure 1.

We aim to integrate study participation as much as possible with the routine care provided at the SCC. Whether or not a patient is willing to participate in the study will not have any influence on the routine care she receives during follow-up.

Study implementation

If the patient is interested to participate in the SOPHIA-study, the treating physician will hand out an invitation for the SOPHIA-study, together with a patient information letter and an informed consent form. After one or two weeks, the treating physician or research nurse will contact the patient by telephone to answer any remaining questions. If the woman agrees to participate, she will be asked to return the signed informed consent form, and subsequently the 'General questionnaire' and 'Menopause questionnaire' (if applicable) will be sent to her home. Patients will be asked to bring their completed questionnaires with them to a follow-visit at the SSC or to return the questionnaires by mail.

The neurocognitive, cardiovascular and BMD assessments will be performed during a follow-up visit at the SCC. Ideally, all medical tests will be performed during two to three follow-up visits, depending on availability and timing of other routine medical care tests (e.g. breast cancer screening). The planned tests with allocated time are shown in Figure 1.

Patients will be tested for renal failure before undergoing the CCTA. Women with severe renal insufficiency, defined as a e-GFR value of <60 ml per minute per 1.73m^2, will undergo a computed tomography coronary calcium score without contrast fluid.

Blood will be drawn at two time points. The first blood sample will be taken at the SCC during a routine care blood withdrawal. The second blood sample will be drawn in fasting state before the CCTA. Patients are offered the possibility to perform the neurocognitive tests at home. In that case, a separate appointment will be made. To ensure sufficient time between two memory tests, patients will be asked to complete the FFQ during this appointment.

Statistical issues

Power calculation

Approximately 500 women are eligible for participation in the three selected hospitals. Based on our previous studies we expect that 60% of the eligible women will participate in the current study (N=300). Power calculations were performed separately for the outcomes BMD, cardiovascular status, neurocognitive function

and QoL. When comparing BMD of women who developed POI (expected N=60 (20%)) with those who did not (expected N=240), there is over 80% power to detect a difference of 0.05 g/cm2 in BMD (1.00 (SD 0.1) vs 1.05 (SD 0.1)).

The power calculation for cardiovascular status is based on the IMT measurement. There is over 80% power to detect a difference of 0.1 mm in mean IMT between women with POI and women with normal menopausal ages (0.6 (SD 0.2) versus 0.5 (SD 0.2). An increase of 0.1 mm in IMT has been associated with an increase in risk of 12% for myocardial infarction.[74]

Previous retrospective neuropsychological studies, in which effects of systemic cancer treatments and POI on cognitive functioning were examined, have yielded significant findings with somewhat smaller group sizes of 39 and 53 patients.[90-92]

For the outcome QoL, we followed the calculations of Cohen.[93] The proposed study has over 80% power to detect moderate difference between women who developed POI and those who did not. All power calculations used a 5% chance for a Type-1 error and a minimal effect size of 0.5.

Statistical analyses

Outcomes of female HL survivors who developed POI will be compared with those of female HL survivors who did not by using chi square tests or Fisher's exact tests (categorical variables) and two tailed t-tests (continuous variables) after appropriate transformation, if necessary. Multivariate regression analyses will be used to examine the effects of POI, menopausal age and HRT use on the primary outcome variables (BMD, cardiovascular status and neurocognitive function) and to assess the effect of these primary outcome variables on QoL. Cox regression models with age as a time scale will be used to examine the independent effect of age at HL treatment and age at developing POI. All analyses will be adjusted for confounders (HL treatment regimen, lifestyle factors, reproductive factors, climacteric symptoms, medication) where applicable. Effect modification and mediation will be tested using interaction terms. We will also perform subgroup analyses to evaluate the difference between a CT- and RT-induced POI in more detail and we will compare cardiovascular status between HL survivors with POI and other women with natural POI. P values <0.05 will be considered statistically significant.

DISCUSSION

In the current study, the long-term effects of CT- and/or RT-induced POI on BMD, cardiovascular status,

neurocognitive function and QoL will be examined by measurements within a cohort of female HL survivors. Approximately 30-40% of the female survivors in our HL cohort treated between 1965 and 1985 experienced treatment-induced POI. The majority of these women have now been postmenopausal for over 20 to 30 years which enables us to examine the very late effects of POI.

Treatment-induced POI might put female HL survivors at high risk for developing adverse POI-associated conditions such as osteoporosis, neurocognitive dysfunction and CVD, while they already have an increased risk for late adverse effects due to the HL treatment itself.[4 9 21 37 46] This may lead to problems in daily functioning and can have a large impact on their QoL, in particular because the conditions may occur at very young ages. Results of this study will help to identify those HL survivors who are at increased risk for osteoporosis, neurocognitive dysfunction and possibly CVD due to treatment-induced POI. This enables timely referral of high-risk women for interventions, thereby reducing (subclinical) adverse events and improving QoL. Moreover, by identifying these long-term risks, physicians can better inform women with POI in the future. Findings of this study will also be relevant for other female cancer patients who received gonadotoxic treatment at premenopausal ages. CT is a major contributor to the development of POI and its use has intensified considerably over the years in many malignancies.[94 95] Therefore, it is expected that the occurrence of POI-associated adverse effects will increase in female cancer survivors in the near future. Since HRT has become subject of much debate in recent years, investigating the effects of HRT on POI-associated conditions will produce valuable knowledge with regard to the HRT-suppletion policy for female cancer survivors in the Netherlands.

Finally, this study provides a unique possibility to challenge the conventional view that reproductive hormone deprivation in females is of key importance in CVD development. This is relevant in the light of other hypotheses that general biological aging mechanisms underlie a combination of POI and CVD, or that CVD risk factors determine age at menopause (reverse causality hypothesis).[42] Comparison of cardiovascular status between women with POI after HL treatment and women with natural POI will allow examination of a cause-effect relationship between early menopause and CVD. We will be able to adjust for the potential effects of HL treatment on CVD risk, as extensive data on HL treatment regimens are available within our cohort.

This study has several notable strengths and limitations. First, this study is embedded in an infrastructure of several multidisciplinary SCCs, enabling a broad scope of medical tests and extensive follow-up care. Detailed treatment and reproductive data will be available from medical records and patient questionnaires. Second, women received a variety of treatments and have long-term follow-up, rendering it possible to examine the

effects of different HL treatments, POI and menopausal age on several outcomes. A limitation of the study includes patient selection. Due to the high risk of late adverse effects in HL survivors, some women are already deceased or not able to participate in the SOPHIA-study. Moreover, some women are under surveillance in a local hospital rather than their original HL treatment centre, while women who are (feeling) healthy may not visit the SCC because of medical costs and/or other obligations (e.g., work, family). We will account for this by obtaining medical data for women under surveillance in others hospitals, and we have near complete data on important competing late adverse effects such as CVD, second malignancies and vital status, as this study is nested within an existing cohort.

Another limitation is that this study is dependent on routine care procedures of the participating SSCs. Therefore, differences between SSCs may occur regarding available equipment and registration of results from medical tests. In addition, the time interval between two medical tests may be variable between patients due to planning issues (e.g., long waiting lists, the aim to plan multiple medical tests in one day), or because one medical test has already been performed in the past year and will not be repeated. We made a standardized abstraction form to ensure all relevant data are gathered and differences between timing of medical tests and hospitals will be accounted for in the analyses.

In conclusion, this article describes the study protocol of the SOPHIA-study which aims to increase knowledge about BMD, cardiovascular status and neurocognitive function in long-term female HL survivors with and without treatment-induced POI, and the potential influence of these long-term effects on QoL. Results of this study will lead to the identification of those HL survivors who are at increased risk for osteoporosis, neurocognitive dysfunction and possibly CVD due to treatment-induced POI. This enables timely referral of high-risk women for interventions, thereby reducing (subclinical) adverse events and improving QoL. Furthermore, results will shed light on existing hypotheses regarding the association between POI and CVD risk. Moreover, HL survivors or other cancer survivors who will experience treatment-induced POI in the future can be better informed about potential long-term effects. Finally, prospective follow-up of the study population will provide insight into longitudinal changes in risk factors and study outcomes.

AUTHORS' CONTRIBUTIONS

The study protocol has been written by IK, AOvW, BA, EvD-dB and FvL. All authors contributed to study design. MH provided statistical advice. JZ, LD, and BA are responsible for patient accrual and inclusion, and JZ, YA, SB, LM, ES, CL, PL, and LD and BA are responsible for the assessment of the outcome variables.

FvL is the principal investigator and responsible for the funding of the study.

All authors revised the manuscript critically for intellectual content and have approved the final manuscript.

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COMPETING INTERESTS

JZ declares she has conducted a research project funded by Roche in the past two years (unrelated to the current study). CBL's department of Reproductive Medicine has received educational and research grants from Merck Serono, Ferring and Auxogyn, and he received speakers fees from MSD, Merck Serono, Ferring and Auxogyn. He is also a consultant for Ferring. PL provided advice to Friesland Campina. All other authors declare no competing interests.

ETHICS AND DISSEMINATION

This study has been approved by the Institutional Review Board of the Netherlands Cancer Institute and has been registered at "Toetsingonline" from the Dutch Central Committee on Research involving Human Subjects (file number NL44714.031.13). Reporting of serious adverse events was exempted for this study, as the burden and risks associated with participation are very low and in accordance with routine medical care. Results will be disseminated through peer-reviewed publications and will be incorporated in follow-up guidelines for HL survivors.

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FIGURE LEGENDS

Figure 1. Study procedures and patient burden, stratified by medical tests for routine care and research

Abbreviations: RT = radiotherapy; CT = chemotherapy; BETTER = Better care after Hodgkin lymphoma, Evaluation of long-Term Treatment Effects and screening Recommendations; CRP = C-reactive protein; BMD = bone mineral density; DEXA = Dual Energy X-ray Absorptiometry; POI = primary ovarian insufficiency

[a] Expected for > 90% of women [b] Expected for 15-40% of women

In case criteria for care are not fulfilled, tests will be performed for research purpose

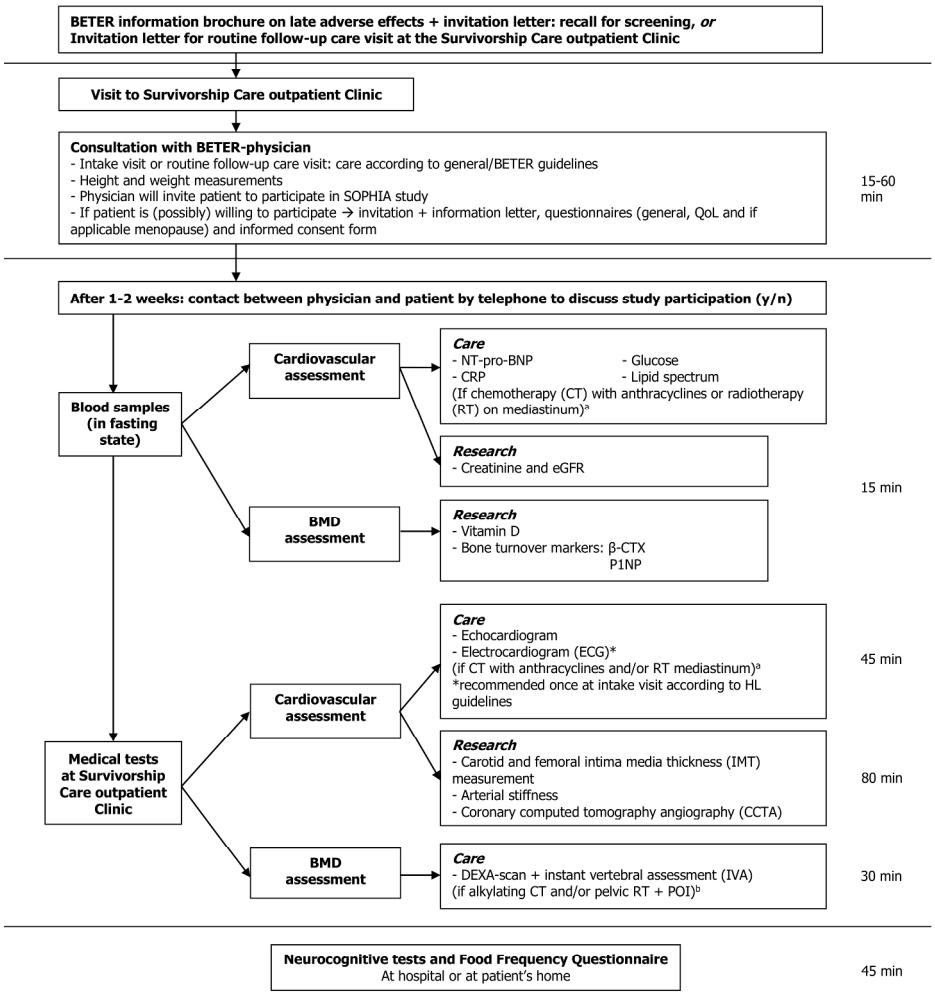

Figure 1. Overview of study procedures and patient burden, stratified by medical tests for routine care and research

279x361mm (300 x 300 DPI)

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Rationale and design of a cohort Study On Primary ovarian insufficiency in female survivors of Hodgkin lymphoma: Influence on long-term Adverse effects (SOPHIA)

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Rationale and design of a cohort Study On Primary ovarian insufficiency in female survivors of Hodgkin lymphoma: Influence on long-term Adverse effects (SOPHIA)

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ABSTRACT

Introduction: Hodgkin lymphoma (HL) has become the prototype of a curable disease. However, many young survivors suffer from late adverse effects of treatment. Both chemotherapy (CT) and radiotherapy (RT) may induce primary ovarian insufficiency (POI), which has been associated with reduced bone mineral density (BMD), neurocognitive dysfunction and possibly cardiovascular disease (CVD). While the general assumption is that POI increases CVD risk, other hypotheses postulate reverse causality, suggesting that cardiovascular risk factors determine menopausal age or that biological aging underlies both POI and CVD risk. None of these hypotheses are supported by convincing evidence. Furthermore, most studies on POI-associated conditions have been conducted in women with early natural or surgery-induced menopause with short follow-up times. In this study we will examine the long-term effects of CT- and/or RT-induced POI on BMD, cardiovascular status, neurocognitive function and quality of life (QoL) in female HL survivors.

Methods and analysis: This study will be performed within an existing Dutch cohort of HL survivors. Eligible women were treated for HL at ages 15-39 years in three large hospitals since 1965 and survived for ≥8 years after their diagnosis. Women visiting a survivorship care outpatient clinic (SSC) will be invited for a neurocognitive, cardiovascular and BMD assessment, and asked to complete several questionnaires and to provide a blood sample. Using multivariable regression analyses we will compare the outcomes of HL survivors who developed POI with those who did not. Cardiovascular status will also be compared with women with natural POI.

Ethics and dissemination: This study has been approved by the Institutional Review Board of the Netherlands Cancer Institute and has been registered at "Toetsingonline" from the Dutch Central Committee on Research involving Human Subjects (file number NL44714.031.13). Results will be disseminated through peer-reviewed publications and will be incorporated in follow-up guidelines for HL survivors.

STRENGHTS AND LIMITATIONS

- This study is the first to examine to the long-term effects of chemotherapy- and radiotherapy -induced primary ovarian insufficiency in female Hodgkin lymphoma survivors
- Furthermore, this study is embedded in an infrastructure of several multidisciplinary survivorship care clinics, enabling a broad scope of medical tests and extensive follow-up care

- Results of this study may help to identify and timely refer those Hodgkin lymphoma survivors who are at increased risk for osteoporosis, neurocognitive dysfunction and possibly cardiovascular disease due to treatment-induced primary ovarian insufficiency for interventions in order to reduce morbidity and enhance quality of life
- Moreover, this study sheds light on different hypotheses regarding the association between primary ovarian insufficiency and cardiovascular disease risk
- Data collection is dependent on routine care procedures of the participating survivorship care clinics and of visiting patients

KEY WORDS

Hodgkin lymphoma, cancer survivor, primary ovarian insufficiency, bone mineral density, cardiovascular disease, neurocognitive function

BACKGROUND

Primary ovarian insufficiency in Hodgkin lymphoma survivors

Due to the improvements in treatment since 1960, Hodgkin lymphoma (HL) has become the prototype of a curable malignancy. Nowadays, overall 10-year survival rates exceed 80%.[1-3] However, survivors are faced with several late adverse effects of treatment, such as second malignancies and cardiovascular disease (CVD) [4-10]. Moreover**,** 30-40% of female HL survivors treated before 1985 developed primary ovarian insufficiency (POI) (menopause before the age of 40 years) [11-15], compared with 1% of women in the general population [16]. Women treated with more recent, less gonadotoxic treatment regimens are likely to have a lower risk, but the long-term risk has not yet been quantified sufficiently.

The risk of POI strongly depends on type of HL treatment, with the highest cumulative risks reported following pelvic RT (81%) and alkylating CT (42-60%).[11-15 17] Older age at treatment (up to 40 years) does not appear to increase risk. Although women treated for HL at older age will develop POI sooner after treatment compared with those treated at younger ages, the cumulative incidence at age 40 is nearly equal in both groups.[11 13 14] In our earlier study among female 5-year HL survivors treated between 1965 and 1995, women developed POI at a median age of 33 years (range 19-39 years).[11] POI occurring this early potentially has a large impact on quality of life (QoL) as it results in infertility, and menopausal symptoms including hot flushes, vaginal dryness and mood swings that are more severe than after menopause at later ages.[18-20] In addition, POI has been associated with reduced bone mineral density (BMD), and increased risk of CVD and neurocognitive dysfunction.

Bone mineral density

Women who reach menopause early (before age 45 years), have a lower BMD and higher incidence of osteoporosis than women who enter menopause at ages ≥50 years.[21-23] Moreover, an early menopause has been associated with a 1.5-3-fold increased fracture risk.[21 22 24] The most common osteoporotic fractures in postmenopausal women occur in the hip, wrist and spine.[24 25] However, it is unclear whether the association between early menopause and BMD and fracture risk persists over time. Some studies have shown that the association becomes much weaker with increasing age (mainly above age 70) [26-28], while others reported a lifetime increased fracture risk.[22 29] Possibly, not only oestrogens but also aging mechanisms are of importance in BMD status.[30]

So far, many studies have been conducted among breast cancer survivors or in women with an early natural or surgery-induced menopause, while studies evaluating the long-term effects of CT- and/or RT-induced POI on

BMD and fracture risk are limited. Two small studies among HL survivors reported a significantly reduced BMD after treatment-induced POI [31 32], while another study found no association.[33] Since HL survivors develop POI at a younger age than breast cancer survivors or the general population, more research is needed to identify the extent of reduced BMD and prevalence of osteoporotic fractures among female HL survivors who developed POI.

Cardiovascular disease

In the general population, early menopause has been associated with an increased incidence of CVD [34-36]. A recent meta-analysis of CVD risk among women with POI showed a pooled HR of 1.6 for total CVD and 1.7 for ischemic heart disease when compared with menopause at ages ≥50 years.[37] Moreover, epidemiological data show a 2% decrease in cardiovascular mortality for each year menopause is delayed.[38] Low levels of testosterone in women have also been associated with increased intima media thickness (IMT) of the carotid artery.[39-41]

An intriguing hypothesis postulates that reverse causality may operate, i.e., that CVD risk factors such as weight, cholesterol and blood pressure determine menopausal age. This is in contrast with the general assumption that endocrine changes due to early menopause are responsible for CVD development. Indeed, in the Framingham Heart Study cohort, a 1% higher premenopausal cardiovascular risk score was associated with a subsequent decrease in menopausal age of 1.8 years.[42] However, it has also been suggested that several risk factors are associated with both CVD and early menopause. A meta-analysis of 22 genome-wide association studies (GWAS) on natural early menopause revealed predominantly genes that are involved in general repair mechanisms [43], arguing for a role of generalized aging rather than ovarian dysfunction in early menopause.

To date, the important question whether accelerated biological aging underlies both early menopause and an increased CVD risk is unresolved and no recent evidence has been provided to support the reverse causality hypothesis. Since POI among HL survivors is induced by exogenous factors (i.e. HL treatment) rather than by endogenous factors (i.e. natural early depletion of the primordial follicle pool) occurring in women with natural POI, a direct comparison between HL survivors and women with natural POI might provide new insights into the association between POI and CVD. If POI would increase CVD risk, this should be considered in the light of an established increased risk of CVD due to mediastinal RT and anthracycline-containing CT.[9]

Neurocognitive function

Although in-vitro studies suggest a neuroprotective effect of oestrogen in the brain, the influence of decreased estrogen levels on cognitive performance is still unclear. Some in-vivo studies have shown an increased risk of neurocognitive impairment or dementia after a surgery-induced early menopause (approximately 1.5-fold), while others found no association.[44-48] These contradicting findings may be due to differences in the cognitive domains that were evaluated, as not all cognitive functions are equally influenced by oestrogens. Hormonal influences seem to mainly concern aspects of memory, information processing speed and executive functioning.[49 50]

Up to now, the long-term effects of POI on cognition are largely unknown, as most studies had short follow-up times and included only women with menopausal ages above 40 years and/or women who used hormone replacement therapy (HRT). Moreover, the majority of studies looked at the effects after oophorectomy, characterized by an abrupt drop of oestrogen levels, while oestrogen levels may decrease gradually in CT-induced POI occurring many years after treatment.[20 51] Preliminary data on POI in HL survivors within our cohort show that women who developed POI had a median duration of ovarian function after HL treatment of 4 years (interquartile range 1-10 years).

Hormone replacement therapy

Much debate surrounds the use of HRT since the Women's Health Initiative (WHI) study reported increased risks of breast cancer, CVD and cognitive impairment after oestrogen and progestin suppletion.[52] More recent studies suggest that the benefit of oestrogen suppletion strongly depends on starting age and timing with respect to menopause.[53-56] Among HL survivors in the Netherlands, HRT has been mainly prescribed to relieve menopausal symptoms and to prevent osteoporosis.[57] However, in several HL treatment centres, HL survivors have been advised to refrain from using HRT against menopausal symptoms because of a potential increase in breast cancer risk.[58] This provides the unique opportunity to examine the effects of HRT use in this population as the long-term effects of HRT on BMD, CVD and neurocognitive function in HL survivors with POI have not been examined yet.

Aim

This article describes the design and methods of a Study On Primary ovarian insufficiency in female survivors of Hodgkin lymphoma: Influence on long-term Adverse effects (SOPHIA). The primary aim of this study is to examine the long-term effects of treatment-induced POI on BMD, cardiovascular status, neurocognitive

function and QoL. We hypothesize that women with treatment-induced POI will have an increased risk of osteoporosis and neurocognitive dysfunction and a lower QoL than HL survivors without POI. However, based on the hypotheses on reverse causality and biological aging, we hypothesize that CVD risk may not be increased in female HL survivors with POI compared to HL survivors without POI.

The secondary aims of this study are:

1. To examine whether long-term effects differ between women with CT- and RT-induced POI
2. To compare cardiovascular status and the possible influence of HRT between HL survivors with treatment-induced POI and women from the general population with natural (non-treatment-induced) POI.

In addition, we will perform exploratory analyses to examine potential differences between subgroups regarding acute (<1 year after HL treatment) and more gradually (≥ 1 year after HL treatment) developed POI and to explore the effects of type and timing of HRT on all outcomes.

METHODS

Design and study population

Hodgkin lymphoma survivors

The SOPHIA-study is an observational cross-sectional study among female HL survivors who are being followed in an outpatient survivorship care clinic. Participants will be invited for a neurocognitive, cardiovascular and BMD assessment and asked to complete several questionnaires and to provide a blood sample. Participants will be recruited from a large previously described cohort of 5-year HL survivors treated in the Netherlands between 1965 and 2000 [4 59], which has been extended with more recently treated patients. Registry data on HL patients treated before 1965 are not available.

This study is a collaboration of three large Dutch Medical Centres: The Netherlands Cancer Institute (NKI), VU University Medical Centre (VUmc) and Leiden University Medical Centre (LUMC). Eligible women were treated for HL at the age of 15-39 years at the adult Haematology-Oncology departments of the three medical centres,. and survived ≥8 years after HL diagnosis. The latter criterion was chosen because we are interested in the long-term effects of POI. Exclusion criteria are: current age of ≥75 years, current treatment for a second malignancy, insufficient understanding of the Dutch language or any psychological, familial, sociological or geographical condition that potentially hampers study participation. General patient characteristics, HL treatment data and follow-up data on vital status, second malignancies and CVD are already available for all 8-year HL-survivors in

these three hospitals, enabling us to monitor possible differences between patients who participate and those who decline. Also, we will be able to examine whether eligible 8-year survivors who died before study invitation died due to one of our outcomes of interest. Due to the high risk of late adverse effects in HL survivors, some women are already deceased. If it would turn out that a relatively large proportion of patients in the POI group (compared to the comparison group) has died of CVD, we will be able to report this, which is a big advantage in a cross-sectional study.

The study has been approved by the Institutional Review Board of The Netherlands Cancer Institute Recruitment started in 2014 (VUmc). We will follow the study population longitudinally to examine changes in risk factor and outcomes over time for which additional funding will be acquired. Moreover, eligible women will be followed through clinical care, where permission is asked to store future blood samples as well.

External control group

To enable the comparison of cardiovascular status between HL survivors and women with natural (non-treatment-induced) POI, we will use data from an ongoing nationwide multicentre study on hypergonadotropic oligomenorrohea/amenorrhea. This study is a collaboration of three University Medical Centres and includes women aged ≥40 years with a diagnosis of polycystic ovarian syndrome (PCOS) or POI between 1992 and 2012. Data collection consists of a cardiovascular risk assessment at the outpatient clinic.[60 61]

Study parameters and data collection

Main outcome measures and other relevant study parameters are briefly described below by method of data collection (see also Table 1). The main exposure POI is defined as amenorrhea for ≥4 months with two serum follicle-stimulating hormone (FSH) levels in the menopausal range (obtained at least 1 month apart), or amenorrhea for ≥12 months before the age of 40 years. In case a woman has already been postmenopausal for many years at study enrolment, POI is defined as the date of or age at last menstruation. Because we performed earlier studies on POI, for the majority of women we already know their menopausal status and age, either from the medical records or from questionnaires sent in the 1990s-2000s. For the remaining women these data will be abstracted from the medical records and/or obtained through the patient questionnaire.

Primary exposure and outcomes	Data collection methods		Outcome variables	Justification of methods
Primary ovarian insufficiency	Blood sample	Hormone level	If indicated for routine care: level of follicle-stimulating hormone (FSH) in mIU/mL	Routine care – diagnostic value
	Questionnaire		Date of last menstruation, menopausal age	
	Medical record		Date of last menstruation, menopausal age	
Bone mineral density	Medical tests	DEXA-scan of lumbar spine and hip by means of Hologic Delphi densitometer (VUmc) or General Electric Scanner (LUMC)	BMD values in g/cm^2 T and Z scores Presence of osteopenia (defined as T-scores of -1 to -2.5) Presence of osteoporosis (defined as T-scores of ≥-2.5)	Routine care – diagnostic value The DEXA-scan is most widely used in clinical practice to screen for osteoporosis and regarded as the 'Golden standard'
		Instant vertebral assessment (IVA) by Hologic Delphi densitometer (VUmc) or General Electric Scanner (LUMC)	Vertebral height reduction in % Presence of clinical and non-clinical vertebral fractures	There is a strong additive value of IVA compared with DEXA alone [62-64]
		Anthropomorphic measurements	Height in cm and weight in kg	
	Blood sample	Bone turnover markers	Bone formation by P1NP - mean value in ng/ml Bone resorption by β-CTX - mean value in pg/ml	These markers have been used in previous studies and are recommended for research purposes [65]
		Vitamin D	Level of 25-hydroxyvitamine D in serum in nmol/l	Vitamin D has been associated with bone turnover markers, BMD, fracture risk and risk of falling [66-68]
	Questionnaire	Food frequency questionnaire (FFQ)	Mean score of calcium intake	The FFQ is a validated questionnaire [69] Reference values are available [70]
		General questionnaire	Previous fractures, use of calcium and vitamin D supplements use of glucocorticoids, family history of osteoporosis	
	Medical record		Earlier DEXA-scans (yes,no), if applicable treatment plan for osteoporosis such as vitamin D supplementation, recommendations for lifestyle changes	
Cardiovascular status	Medical tests	Echocardiogram If contra-indicated: cardiac magnetic resonance imaging (MRI)	Abnormalities in heart structure Left ventricular function (LVF) by E/A ratio, deceleration time, isovolumic relaxation time (IVRT), left ventricular ejection fraction (LVEF), diastolic and systolic diameter and volume, E/e' ratio Right ventricular function: tricuspid annular plane systolic excursion (TAPSE) Presence of mitral, aortic or tricuspid valve defects i.e. insufficiencies or stenoses Wall motion score index	Routine care – diagnostic value
		Electrocardiogram (ECG)	Sinus rhythm, QRS complex, ST morphology (elevation or depression), PQ interval and left ventricle hypertrophy	Routine care – diagnostic value
		Coronary computed tomography angiography (CCTA) by a 320-detector	Coronary artery calcium (CAC) score according to Agatston Presence of luminal narrowing and if applicable: type of	High sensitivity and specificity [71] Most valid alternative method for detecting

		row volumetric scanner (Aquilion ONE) (LUMC) and 256 Scanner Philips (VUmc)	narrowing and number of plaques for the left main coronary artery (LMCA), left anterior descending (LAD), circumflex artery (CRX) and right coronary artery (RCA)	significant coronary disease (golden standard is invasive coronary angiography) [72]
		Vascular measurements	Presence of atherosclerosis by carotid intima media thickness (cIMT) and femoral IMT in mm Arterial stiffness (VUmc only)	Predictors of future cardiovascular events [73-75]
		Blood pressure	Mean of three consecutive measurements in mm/Hg	
		Anthropomorphic measurements	Height in cm, weight in kg, body mass index (BMI) in kg/cm^2, hip circumference in cm, waist circumference in cm, hip-waist ratio	
	Blood sample	Biomarkers	Left ventricular function and presence of ischemia and infarction by NT-pro-BNP in pmol/l Chronic inflammation (associated with atherosclerosis) by CRP in mg/L	In general population: strong predictor of coronary heart disease [76 77]
		Lipid spectrum	Total cholesterol, HDL, LDL, triglycerides	Established risk factors for CVD
		Glucose	Fasting blood glucose	Established risk factor for diabetes
		Kidney function	Creatinine, estimated glomerular filtration rate (eGFR)	Routine care before CCTA
	Questionnaire	'General questionnaire'	(Family) history of CVD and risk factors for CVD and if applicable date of diagnosis and treatment	
	Medical record		Cardiovascular risk score based on SCORE chart and Framingham chart, adjusted for age Cardiovascular history, contra-indications for echocardiogram	
Neurocognitive function	Neurocognitive tests	15 Words test	Verbal memory in total number of words	These tests were selected based on their reliability, validity and availability of reference norms. The domains examined are potentially sensitive for the effect of oestrogens [48,62-67]
		Trail making test A&B	Information processing speed) in seconds to complete	
		COWA verbal fluency test	Verbal fluency in total number of words	
		Letter-number sequencing	Working memory in total correct trials	
		WAIS III Digit span	Measures concentration in total number of items/ lists correctly repeated) can be converted to a scaled score, which is an age-based, norm-referenced score for each subject	
		Dutch Adult Reading Test (NART)	Verbal intelligence in mean IQ estimate	
Quality of life	Questionnaire	SF-12	General health	Shortened version of the validated questionnaire SF-36, which has been previously used in Dutch studies [78]
		MOS cognitive functioning scale	Cognitive functioning	Frequently used questionnaire [79]
		Hospital Anxiety and Depression Scale (HADS)	Anxiety and depression	Valid and reliable Dutch reference values are available [80]
		Sexual Activity Questionnaire (SAQ)	Sexual functioning	The SAQ is a valid, reliable and acceptable measure for describing the sexual functioning of women in terms of activity, pleasure and discomfort. It is quick and easy to administer and has good face validity discriminating

				between the sexual functioning of pre- and post-menopausal women [81]
		Shortened fatigue questionnaire (VVV)	Fatigue	Reliable and validated questionnaire [82]

Table 1. Overview of outcome measures and corresponding data collection methods

Medical records

Data on HL diagnosis (date, pathology), primary and recurrence treatment (including date, RT fields, chemotherapeutic regimens and doses) and follow-up data have been previously collected from medical records [4]. Since treatment for HL has changed considerably over time, a variety of treatment regimens was used. Primary treatment was usually given according to treatment protocols of the European Organization of Research and Treatment of Cancer (EORTC) and German Hodgkin lymphoma Study Group (GHSG), while treatment for recurrences was generally not standardized. Furthermore, data on reproductive factors (e.g., menopausal age) will be obtained.

Patient questionnaire data

Four questionnaires will be used to ascertain data on women's general characteristics, QoL, calcium intake and menopause related topics. The 'General characteristics questionnaire' will obtain information on the following items: reproductive history (e.g., age at menarche and menopause, parity, hormone use), general cardiovascular history, bone health status (e.g., previous fractures, use of medication), and lifestyle factors (e.g. current and previous smoking habits, alcohol use and physical activity). The 'QoL questionnaire' consists of five short validated and/or frequently used questionnaires regarding health, cognition, sexual activity, depression and fatigue (Table 1). A validated food frequency questionnaire (FFQ) will be used to assess calcium intake [69]. Reference values will be obtained from the report on dietary intake by the Health Council of the Netherlands [70]. The 'Menopause questionnaire' is specifically aimed at postmenopausal women and will collect information on climacteric symptoms (i.e., severity and frequency) and changes in lifestyle factors after the onset of menopause. Data regarding infertility issues will be ascertained for women who experienced POI.

Neurocognitive, cardiovascular and fracture risk assessments

For the neurocognitive assessment, we have chosen tests that measure cognitive domains potentially sensitive for effects of oestrogens.[49 83-88] Tests were selected based on their reliability, validity and availability of Dutch reference norms. The cardiovascular assessment includes an echocardiogram, electrocardiogram (ECG), coronary computed tomography angiography (CCTA), and measurement of the carotid and femoral IMT, blood pressure and hip/waist circumference. The BMD assessment consists of a DEXA-scan with instant vertebral assessment (IVA). These medical tests were chosen based on their availability and use in clinical practice and their evidence-based diagnostic or predictive value (Table 1).

Blood samples

A blood sample will be drawn to examine bone turnover (i.e., β-CTX for bone resorption and P1NP for bone formation) and cardiac (e.g. NT-pro-BNP, CRP, lipid spectrum) markers. Since new techniques in this research field develop rapidly, we will collect an additional blood sample for future analyses on new biomarkers, such as biomarkers predictive for late effects. Moreover, this sample will be used for future DNA extraction and analyses (e.g. to examine modifying effects of genetic factors, such as single nucleotide polymorphisms (SNP's) associated with POI). These blood samples will be frozen and stored at -80 °C.

Study procedures

Recruitment

Women will be recruited through the Survivorship Care outpatient Clinic (SCC) for HL survivors, established by the Dutch nationwide BETER consortium (Better care after Hodgkin lymphoma, Evaluation of long-Term Treatment Effects and screening Recommendations). This consortium consists of haematologists and radiation oncologists of >20 hospitals and has developed evidence-based guidelines on follow-up care, including recommendations on cardiovascular risk assessment in order to reduce morbidity and mortality [89]. The three medical centres participating in the SOPHIA-study all have an active SCC where HL survivors can be screened. Approximately 30% of the 5-year HL survivors already receive routine follow-up care on a yearly basis in their original HL treatment centre. All 5-year HL survivors who are currently not under surveillance will be invited for screening (if treated at the age of 15-60 years and currently aged <75 years) by the BETER consortium in the upcoming years.

If a woman, eligible for the SOPHIA-study, visits the SCC of NKI, LUMC or VUmc (either during an intake or follow-up care visit), she will be invited by her treating physician. As mentioned above, follow-up care is provided according to the BETER guidelines and depends on the specific treatments a patient received (e.g., chest or pelvic RT, anthracycline-containing CT). As some of the medical tests in the SOPHIA-study are incorporated in the BETER guidelines, these tests may be part of routine care. Therefore, the physician will determine for each patient which medical tests will be performed for routine care and which will be additionally performed for research purposes. Tests that are considered for routine care may have been recently performed in a participant. If there is no clinical reason to repeat the test, the result of the previous test will be abstracted from the medical record. More details on the distinction between routine care and research tests, and the study procedures are described in Figure 1.

We aim to integrate study participation as much as possible with the routine care provided at the SCC. Whether or not a patient is willing to participate in the study will not have any influence on the routine care she receives during follow-up.

Study implementation

If the patient is interested to participate in the SOPHIA-study, the treating physician will hand out an invitation for the SOPHIA-study, together with a patient information letter and an informed consent form. After one or two weeks, the treating physician or research nurse will contact the patient by telephone to answer any remaining questions. If the woman agrees to participate, she will be asked to return the signed informed consent form, and subsequently the 'General questionnaire' and 'Menopause questionnaire' (if applicable) will be sent to her home. Patients will be asked to bring their completed questionnaires with them to a follow-visit at the SSC or to return the questionnaires by mail.

The neurocognitive, cardiovascular and BMD assessments will be performed during a follow-up visit at the SCC. Ideally, all medical tests will be performed during two to three follow-up visits, depending on availability and timing of other routine medical care tests (e.g. breast cancer screening). The planned tests with allocated time are shown in Figure 1.

Patients will be tested for renal failure before undergoing the CCTA. Women with severe renal insufficiency, defined as a e-GFR value of <60 ml per minute per 1.73m^2, will undergo a computed tomography coronary calcium score without contrast fluid.

Blood will be drawn at two time points. The first blood sample will be taken at the SCC during a routine care blood withdrawal. The second blood sample will be drawn in fasting state before the CCTA. Patients are offered the possibility to perform the neurocognitive tests at home. In that case, a separate appointment will be made. To ensure sufficient time between two memory tests, patients will be asked to complete the FFQ during this appointment.

Patient and public involvement

This study was designed in collaboration with several physicians working at the Survivorship Care outpatient Clinic (BETER). They provided relevant insights into so far unrecognized health and psychosocial issues encountered by HL survivors, which were incorporated in both our research questions and our study procedures.

Moreover, before the start of the study, we presented the study design and procedures at a Survivorship meeting for patients from the Dutch Lymphoma Association. We asked for feedback and five female HL survivors volunteered to review our questionnaires and study information letters. Several changes were made following their comments. These women were also involved in the decision-making regarding the name and logo of the study.

Results from the study will be disseminated through the Dutch website for HL survivors: www.beternahodkgin.nl and the Dutch Lymphoma Association.

Statistical issues

Power calculation

Approximately 500 women are eligible for participation in the three selected hospitals. Based on our previous studies we expect that 60% of the eligible women will participate in the current study (N=300). Power calculations were performed separately for the outcomes BMD, cardiovascular status, neurocognitive function and QoL. However, conclusions about associations between POI and the different outcomes will not be based on a single test but on how plausible a true association is given the results of the various analyses specified. Instead of formally adjusting p-values for multiple comparisons, we will consider the possibility of a type 1 error in our interpretation of the results for each outcome.

When comparing BMD of women who developed POI (expected N=60 (20%)) with those who did not (expected N=240), there is over 80% power to detect a difference of 0.05 g/cm2 in BMD (1.00 (SD 0.1) vs 1.05 (SD 0.1)).

The power calculation for cardiovascular status is based on the IMT measurement. There is over 80% power to detect a difference of 0.1 mm in mean IMT between women with POI and women with normal menopausal ages (0.6 (SD 0.2) versus 0.5 (SD 0.2). An increase of 0.1 mm in IMT has been associated with an increase in risk of 12% for myocardial infarction.[74]

Previous retrospective neuropsychological studies, in which effects of systemic cancer treatments and POI on cognitive functioning were examined, have yielded significant findings with somewhat smaller group sizes of 39 and 53 patients.[90-92]

For the outcome QoL, we followed the calculations of Cohen.[93] The proposed study has over 80% power to detect moderate difference between women who developed POI and those who did not. All power calculations used a 5% chance for a Type-1 error and a minimal effect size of 0.5.

Statistical analyses

Characteristics of female HL survivors who developed POI will be compared with those of female HL survivors who did not by using chi square tests or Fisher's exact tests (categorical variables) and two tailed t-tests (continuous variables) after appropriate transformation, if necessary. Multivariate regression analyses will be used to examine the effects of POI, menopausal age and HRT use on the primary outcome variables (BMD, cardiovascular status and neurocognitive function) and to assess the effect of these primary outcome variables on QoL. Cox regression models with age as a time scale will be used to examine the independent effect of age at HL treatment on age at developing POI. Potential confounders (HL treatment, lifestyle factors, reproductive factors, climacteric symptoms, and medications) will be added one by one to models with POI and the different main outcome variables to determine whether they are confounding the POI effect. Propensity score analyses will be used instead of adjustment if the number of confounders is large. Effect modification and mediation will be tested using interaction terms. We will also perform subgroup analyses to evaluate the difference between a CT- and RT-induced POI in more detail and we will compare cardiovascular status between HL survivors with POI and other women with natural POI. P values <0.05 will be considered statistically significant.

DISCUSSION

In the current study, the long-term effects of CT- and/or RT-induced POI on BMD, cardiovascular status, neurocognitive function and QoL will be examined by measurements within a cohort of female HL survivors. Approximately 30-40% of the female survivors in our HL cohort treated between 1965 and 1985 experienced treatment-induced POI. The majority of these women have now been postmenopausal for over 20 to 30 years which enables us to examine the very late effects of POI.

Treatment-induced POI might put female HL survivors at high risk for developing adverse POI-associated conditions such as osteoporosis, neurocognitive dysfunction and CVD, while they already have an increased risk for late adverse effects due to the HL treatment itself.[4 9 21 37 46] This may lead to problems in daily functioning and can have a large impact on their QoL, in particular because the conditions may occur at very young ages. Results of this study will help to identify those HL survivors who are at increased risk for osteoporosis, neurocognitive dysfunction and possibly CVD due to treatment-induced POI. This enables timely referral of high-risk women for interventions, thereby reducing (subclinical) adverse events and improving QoL. Moreover, by identifying these long-term risks, physicians can better inform women with POI in the future. Findings of this study will also be relevant for other female cancer patients who received gonadotoxic

treatment at premenopausal ages. CT is a major contributor to the development of POI and its use has intensified considerably over the years in many malignancies.[94 95] Therefore, it is expected that the occurrence of POI-associated adverse effects will increase in female cancer survivors in the near future. Since HRT has become subject of much debate in recent years, investigating the effects of HRT on POI-associated conditions will produce valuable knowledge with regard to the HRT-suppletion policy for female cancer survivors in the Netherlands.

Finally, this study provides a unique possibility to challenge the conventional view that reproductive hormone deprivation in females is of key importance in CVD development. This is relevant in the light of other hypotheses that general biological aging mechanisms underlie a combination of POI and CVD, or that CVD risk factors determine age at menopause (reverse causality hypothesis).[42] Comparison of cardiovascular status between women with POI after HL treatment and women with natural POI will allow examination of a cause-effect relationship between early menopause and CVD. We will be able to adjust for the potential effects of HL treatment on CVD risk, as extensive data on HL treatment regimens are available within our cohort.

This study has several notable strengths and limitations. First, this study is embedded in an infrastructure of several multidisciplinary SCCs, enabling a broad scope of medical tests and extensive follow-up care. Detailed treatment and reproductive data will be available from medical records and patient questionnaires. Second, women received a variety of treatments and have long-term follow-up, rendering it possible to examine the effects of different HL treatments, POI and menopausal age on several outcomes. A limitation of the study includes patient selection. Due to the high risk of late adverse effects in HL survivors, some women are already deceased or not able to participate in the SOPHIA-study. Moreover, some women are under surveillance in a local hospital rather than their original HL treatment centre, while women who are (feeling) healthy may not visit the SCC because of medical costs and/or other obligations (e.g., work, family). We will account for this by obtaining medical data for women under surveillance in others hospitals, and we have near complete data on important competing late adverse effects such as CVD, second malignancies and vital status, as this study is nested within an existing cohort. This also allows us to evaluate potential differences in disease risks between participants and our entire cohort in order to quantify any survivorship bias and to adequately interpret the strength of our results.

Another limitation is that this study is dependent on routine care procedures of the participating SSCs. Therefore, differences between SSCs may occur regarding available equipment and registration of results from medical tests. In addition, the time interval between two medical tests may be variable between patients due

to planning issues (e.g., long waiting lists, the aim to plan multiple medical tests in one day), or because one medical test has already been performed in the past year and will not be repeated. We made a standardized abstraction form to ensure all relevant data are gathered and differences between timing of medical tests and hospitals will be accounted for in the analyses.

In conclusion, this article describes the study protocol of the SOPHIA-study which aims to increase knowledge about BMD, cardiovascular status and neurocognitive function in long-term female HL survivors with and without treatment-induced POI, and the potential influence of these long-term effects on QoL. Results of this study will lead to the identification of those HL survivors who are at increased risk for osteoporosis, neurocognitive dysfunction and possibly CVD due to treatment-induced POI. This enables timely referral of high-risk women for interventions, thereby reducing (subclinical) adverse events and improving QoL. Furthermore, results will shed light on existing hypotheses regarding the association between POI and CVD risk. Moreover, HL survivors or other cancer survivors who will experience treatment-induced POI in the future can be better informed about potential long-term effects. Finally, prospective follow-up of the study population will provide insight into longitudinal changes in risk factors and study outcomes.

AUTHORS' CONTRIBUTIONS

The study protocol has been written by IK, AOvW, BA, EvD-dB and FvL. All authors contributed to study design. MH is involved as statistician and performed the sample size calculations together with IK. JZ, LD, and BA are responsible for patient accrual and inclusion, and JZ, YA, SB, LM, ES, CL, PL, and LD and BA are responsible for the assessment of the outcome variables.

FvL is the principal investigator and responsible for the funding of the study.

All authors revised the manuscript critically for intellectual content and have approved the final manuscript.

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COMPETING INTERESTS

JZ declares she has conducted a research project funded by Roche in the past two years (unrelated to the current study). CBL's department of Reproductive Medicine has received educational and research grants from Merck Serono, Ferring and Auxogyn, and he received speakers fees from MSD, Merck Serono, Ferring and

Auxogyn. He is also a consultant for Ferring. PL provided advice to Friesland Campina. All other authors declare no competing interests.

ETHICS AND DISSEMINATION

This study has been approved by the Institutional Review Board of the Netherlands Cancer Institute and has been registered at "Toetsingonline" from the Dutch Central Committee on Research involving Human Subjects (file number NL44714.031.13). Reporting of serious adverse events was exempted for this study, as the burden and risks associated with participation are very low and in accordance with routine medical care. Results will be disseminated through peer-reviewed publications and will be incorporated in follow-up guidelines for HL survivors.

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FIGURE LEGENDS

Figure 1. Study procedures and patient burden, stratified by medical tests for routine care and research

Abbreviations: RT = radiotherapy; CT = chemotherapy; BETTER = Better care after Hodgkin lymphoma, Evaluation of long-Term Treatment Effects and screening Recommendations; CRP = C-reactive protein; BMD = bone mineral density; DEXA = Dual Energy X-ray Absorptiometry

[a] Expected for > 90% of women [b] Expected for 15-40% of women

In case criteria for care are not fulfilled, tests will be performed for research purpose

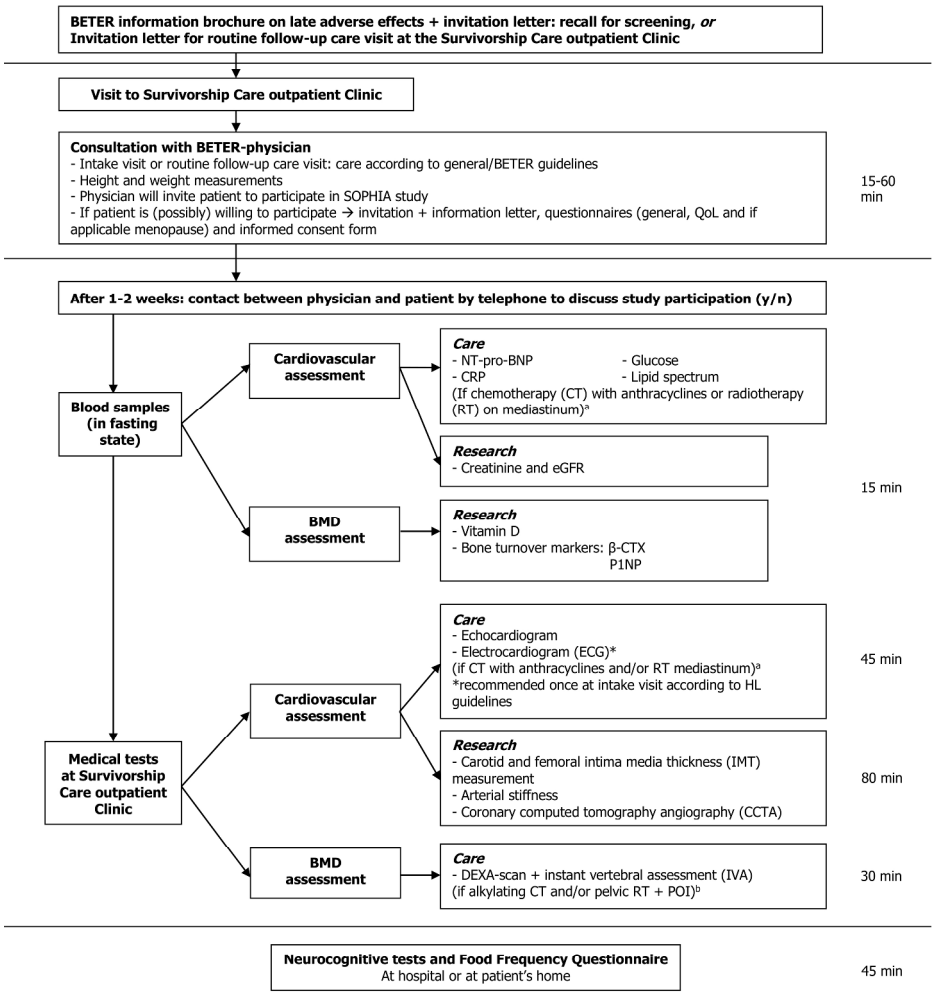

Figure 1. Overview of study procedures and patient burden, stratified by medical tests for routine care and research

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