Growth Factors

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/igrf20

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To cite this article: Karin Larsson, Martin Höglund, Anders Larsson, Måns Thulin & Torbjörn Karlsson (2020) Increased levels of the cardiovascular disease risk biomarkers GDF15 and myostatin in patients with chronic lymphocytic leukemia, Growth Factors, 38:3-4, 189-196, DOI: 10.1080/08977194.2021.1932870

To link to this article: https://doi.org/10.1080/08977194.2021.1932870

GROWTH FACTORS
2020, VOL. 38, NOS. 3–4, 189–196
https://doi.org/10.1080/08977194.2021.1932870

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ARTICLES

Increased levels of the cardiovascular disease risk biomarkers GDF15 and myostatin in patients with chronic lymphocytic leukemia

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ABSTRACT

Individuals suffering from cancer, including hematological malignancies, are at increased risk of cardiovascular disease (CVD). Elevated levels of several biomarkers in blood are associated with an increased risk of CVD. The aim of this study was to investigate whether a subset of such CVD risk biomarkers was elevated in patients with untreated chronic lymphocytic leukemia (CLL). Blood plasma and serum from 139 CLL patients and 71 healthy age-matched controls were analyzed for 11 proposed CVD risk biomarkers. The CLL cohort displayed a more heterogeneous pattern of biomarker expression compared to controls. The majority, eight out of 11, analyzed CVD risk biomarkers differed significantly in concentrations between CLL patients and controls. Increased levels of the biomarkers GDF15 and myostatin have not previously been reported in CLL. Further prospective studies are warranted to investigate whether these biomarkers predict future cardiovascular events in patients with CLL.

ARTICLE HISTORY
Received 21 January 2021
Accepted 11 May 2021

KEYWORDS
Chronic lymphocytic leukemia; inflammation biomarkers; cardiovascular disease biomarkers; cytokines

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world. It is a disease of mature, clonal B-cells expressing CD5 and CD19. The median age at diagnosis is 72 years, and the disease is more prevalent in men than in women, with a ratio of 1.6:1 (Mattsson et al. 2020). Taking these characteristics into account, one can assume that a large proportion of CLL patients will present with concurrent cardiovascular disease (CVD).

It is known that chronic inflammation increases the risk of both cancer and CVD (Grivennikov, Greten, and Karin 2010) and it may have a role in the development of CLL (Rozovski, Keating, and Estrov 2013). Although clonal hematopoiesis has been associated with the development of atherosclerosis (Jaiswal et al. 2017), it is unknown whether CLL in itself could promote the progress of atherosclerosis and CVD. Interestingly, in 2019, Strongman et al. Strongman et al. (2019) found an increase in CVD among patients suffering from cancer, including hematological diseases.

In the process of atherosclerotic plaque formation, inflammation plays a central role, and its pathogenesis has several similarities with tumor development, such as defect endothelial barrier function and extracellular matrix remodeling as well as leukocyte infiltration into the vessel wall. Cytokines with pro-inflammatory effects and growth factors, such as interleukin-1b (IL-1b), tumor necrosis factor alpha (TNFα), and vascular endothelial growth factor (VEGF), which are involved in cancer development, also act in the formation of atherosclerotic plaques (Libby and Kobold 2019). Pro-inflammatory cytokines have been found to be elevated in plasma from CLL patients, for example, IL-4, IL-6, IL-8, and TNFα (Grivennikov, Greten, and Karin 2010; Rozovski, Keating, and Estrov 2013).

Recently, a Swedish population-based register study found that one third of all patients suffered from CVD at the time of CLL diagnosis (Larsson et al. 2020). This high CVD prevalence calls for awareness

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A complete list of abbreviations used in this manuscript is enclosed in supplementals.

Supplemental data for this article can be accessed here.

among hematologists and oncologists treating CLL patients with modern targeted therapy such as ibrutinib, a Bruton's tyrosine kinase inhibitor (BTKi), the use of which has increased rapidly due to high efficacy also in patients with poor risk genetics (Byrd et al. 2019). BTKis inhibit transcription factors nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3), among other key components of the inflammatory response. However, BKTis also exhibit off-target effects, leading to an increased rate of adverse events, such as atrial fibrillation and hypertension in the case of ibrutinib treatment. These events are mediated through other kinases, such as inhibition of cardiac phosphoinositide 3-kinase (PI3K), which has been suggested to predispose for atrial fibrillation in animal models. The mechanisms behind hypertension remain to be further investigated (Stephens et al. 2019).

With this background on CLL and CVD, we analyzed the plasma concentration of 11 biomarkers known to be associated with CVD, in a newly diagnosed real-world CLL population. The results were compared with those from healthy controls. Of special interest were GDF-15 and myostatin, since they have not previously been reported in CLL and represent different aspects of the inflammatory process. The majority of the patients had indolent disease. Results were compared with those of healthy controls.

Materials and methods

Study subjects: Uppsala U-CAN CLL biobank

The U-CAN biobank, organized by the Uppsala-Umea Comprehensive Cancer Consortium, is a tumor biobank collecting biological material from cancer patients before, during, and after cancer treatment. In this study, blood plasma and serum from 139 newly diagnosed CLL patients from this biobank was used for analysis of 11 CVD risk biomarkers: B2M and C-reactive protein (CRP), galectin-3, growth differentiation factor 15 (GDF15), matrix metalloproteinase 9 (MMP9), myostatin, pentraxin 3 (PTX3), soluble TNFα receptor 1 and 2 (sTNFR1 and 2), and soluble VEGF receptor 1 and 2 (sVEGFR1 and 2). Blood plasma and serum from 71 age- and sex-matched healthy blood donors were used as controls (ethical approval: Ups 01-367).

The study was approved by the Research Ethics Committee of Uppsala University (Epn 2010/98 and 2014/233).

Laboratory methods

Serum samples were used for analyses of CRP and B2M. CRP (reagent: 6K26, Abbott Laboratories, Abbott Park, IL) and B2M (reagent: Tina-quant β2-Microglobulin, Roche Diagnostics Scandinavia, Bromma, Sweden) were analyzed on a BS380 instrument (Mindray, Shenzhen, China).

Plasma EDTA samples were used for the following analyses: galectin 3 (DY1154), GDF15 (DY957), MMP9 (DY911), myostatin (DGDF80), PTX3 (DY1826), TNFRSF1A (DY225), sTNF RII/TNFRSF1B (DY276), sVEGFR1 (DY321B), and sVEGFR2 (DY357). Analyses were performed using commercial sandwich ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. In a first step, monoclonal antibodies specific for the peptides were coated onto microtiter plates. After blocking the wells with bovine serum albumin, standards and samples were pipetted into the wells and the peptides were bound to the immobilized antibodies. After washing, a biotinylated anti-peptide antibody was added to the wells. After another incubation and washing cycle, a streptavidin-HRP conjugate was added. Finally, after incubation and washing, a substrate solution was added. The development was stopped and the absorbance was measured. Human myostatin was analyzed by a sandwich ELISA (R&D Systems, Minneapolis, MN). Absorbance was measured using a SpectraMax 250 analyzer (Molecular Devices, Sunnyvale, CA). The total coefficient of variations of the assays was approximately 6%. The assays were performed blinded without knowledge of the clinical diagnosis. All samples were analyzed in the same run using two microtiter plates to reduce plate to plate variation.

Data and statistical analysis

Statistical analyses were performed with the software R 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). To test for group differences, linear models adjusted for age and sex were used, using permutation tests to account for non-normality and using the Benjamini–Hochberg procedure to adjust for multiple testing. Principal component analysis was used to visualize group differences.

Results

In total, 139 CLL patients (94 males and 45 females) were included in this study. The median age was 70 and 67 years for the CLL patients and controls,

respectively. All CLL patients were treatment naïve. The proportion of patients in Binet stages A, B, and C was 82%, 10%, and 8%, respectively. The clinical characteristics of the CLL cohort and the controls are presented in Table 1, as are the results for the 11 biomarkers analyzed.

Summarizing the results of the 11 CVD biomarkers in a PCA plot, we observed that the CLL patients expressed a more heterogeneous pattern, whereas the controls clustered more homogeneously (Figure 1).

Table 1. Clinical characteristics of the CLL patients and controls and results of the 11 biomarkers. The CLL patients exhibited higher concentration of all nine biomarkers with significant difference.

	CLL patients, $N = 139$	Controls, $N = 71$
Clinical characteristics		
Age (years in median, range)	70 (37–88)	67 (43–79)
Male (*n*, %)	94 (68)	46 (65)
Female (*n*, %)	45 (32)	25 (35)
Binet (*n*, %)		
A	104 (82)	N/A
B	13 (10)	N/A
C	10 (8)	N/A
Unknown	12	
Biomarker	Difference (s.e.[a])	*p*-value
B2M	0.27 (0.20)	<0.05
CRP	1.21 (1.97)	>0.05
Galectin-3	4188 (854)	<0.001
GDF15	76 (61)	<0.05
MMP9	44 691 (12 427)	<0.001
Myostatin	409 (167)	<0.001
PTX3	527 (802)	>0.05
sTNFR1	551 (133)	<0.001
sTNFR2	1683 (559)	<0.001
sVEGFR1	264 (281)	>0.05
sVEGFR2	849 (174)	<0.001

[a]Standard error.

The levels of the CVD risk biomarkers galectin-3, GDF15, MMP9, myostatin, sTNFr1, sTNFr2, and sVEGFR2 were higher in CLL patients than in controls. No significant difference was seen for CRP, PTX3, or sVEGFr1 between the two groups (Table 1). Figure 2(A,B) demonstrates boxplots for the biomarkers GDF15 and myostatin. Boxplots for the other biomarkers analyzed are presented in the supplements, as are median, mean, maximum, and minimum values for all biomarkers in each group.

Discussion

Summarizing the results of our population-based CLL cohort, we conclude that the CLL population expresses a different pattern of CVD risk biomarkers compared to controls, who clustered more homogeneously. Our findings of increased blood levels of GDF15 and myostatin in CLL are novel. Notably, all analyzed biomarkers are associated with both inflammation and CVD. The latter is of special interest, since CVD is common among CLL patients; a register study of all patients diagnosed with CLL in Sweden during 2007–2010 (Larsson et al. 2020) demonstrated that as many as one third had a history of CVD at the time of CLL diagnosis, and 37% at the time of therapy initiation. However, it remains to be shown that CVD is more common in CLL or whether this high incidence of CVD rather reflects the high median age of the CLL population. Contributing to the increased interest in the CLL and CVD

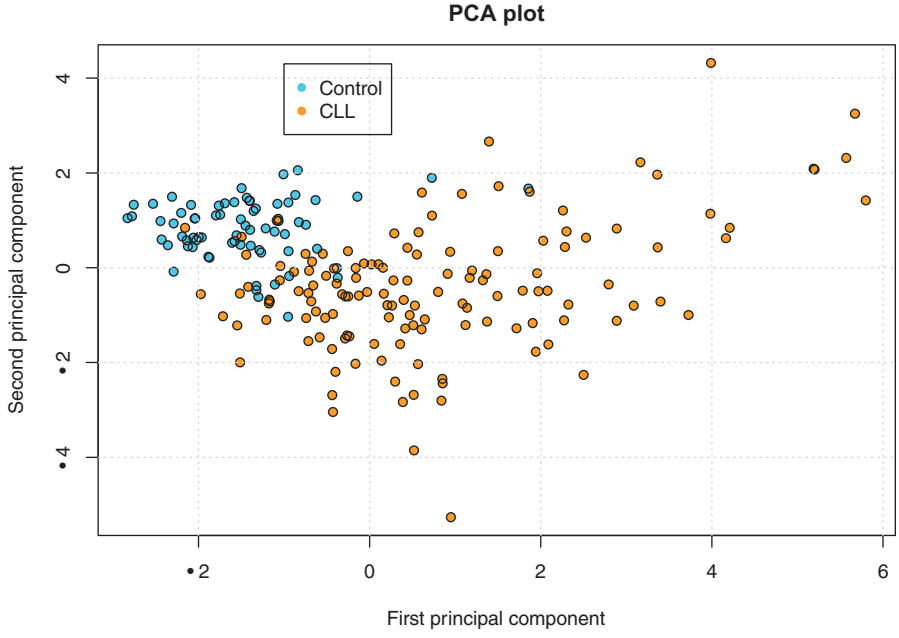

Figure 1. PCA plot showing the heterogeneous expression of biomarkers among the CLL patients in yellow versus controls in blue, who clusters more homogeneously.

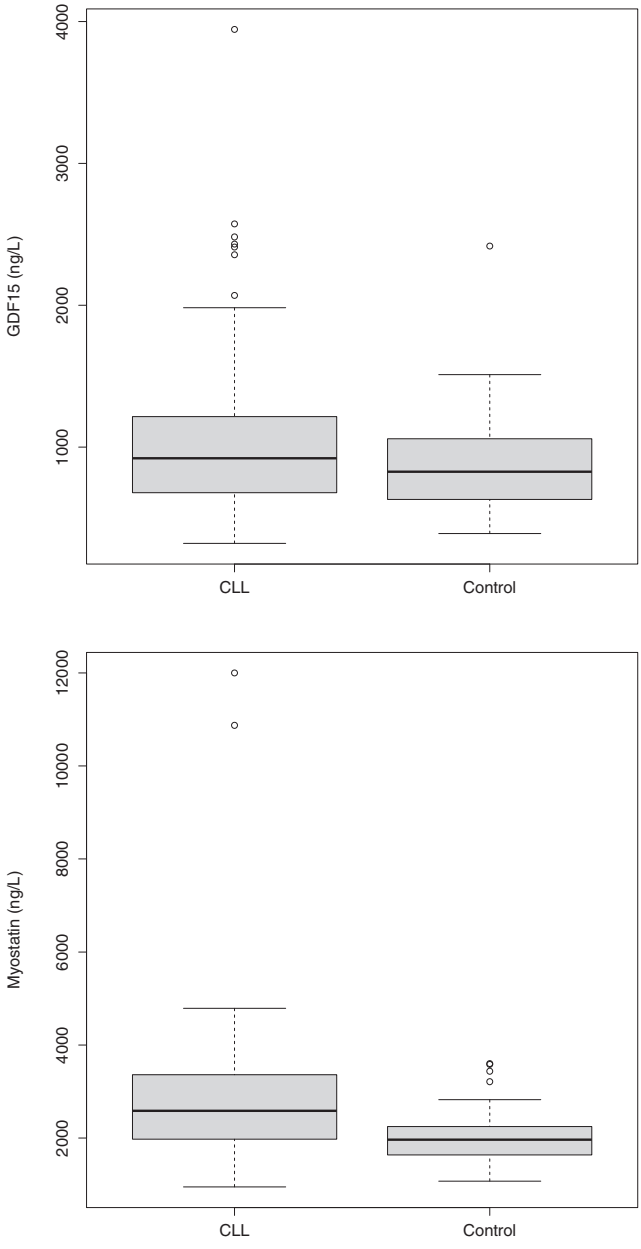

Figure 2. Boxplots visualizing the heterogeneous and diverging appearance in plasma between controls and CLL patients of two cardiovascular biomarkers. (A) Results for GDF-15. (B) Results for myostatin. Medians are shown as thick lines. The bottom and top of the boxes represent the first and third quartiles. The whiskers show the smallest and high non-outliers values. Outliers are shown as circles.

association is the introduction of BTKis, the use of which is associated with hypertension and atrial fibrillation. In this discussion, we primarily focus on discussing our novel results regarding the two biomarkers in CLL mentioned above.

GDF15 is a member of the transforming growth factor β (TGFβ) cytokine superfamily. The protein has been found in several tissues, but with extra enrichment in placenta (Bootcov et al. 1997). High levels of GDF15 in plasma/serum are associated with cancer, inflammation, and CVD (Wollert, Kempf, and Wallentin 2017). Previous data on GDF15 and CLL are lacking. However, patients with multiple myeloma have significantly higher levels of GDF15 in serum compared with healthy controls. Furthermore, patients with myeloma bone disease expressed higher levels than patients with no bone lesions (Westhrin et al. 2015). Similar results were reported by a French study of 131 myeloma patients, demonstrating that patients with high levels of GDF15 in plasma at the time of myeloma diagnosis had a lower probability of event-free and overall survival at 30 months (Corre et al. 2012). Our finding of higher levels of GDF15 in CLL patients compared with healthy controls is in line with previous data on cancer and GDF15. Increased levels of GDF15 have been associated with future events of both cancer and CVD among community-dwelling individuals (Wollert, Kempf, and Wallentin 2017), Andersson et al. (2016) reported that high concentration of GDF15 is related to vascular dysfunction in adults. Moreover, GDF15 interferes with normal vascular function and suppresses angiogenesis in experimental models (Whitson, Lucia, and Lambert 2013) suggesting a link between GDF15 and CVD. Interestingly, *in vivo* results from an experimental study comparing wild-type and GDF15-deficient mice, showed that GDF15 is strongly upregulated following cardiac infarction, and it is suggested to have a cardioprotective effect in regard to ischemia and reperfusion injury (Kempf et al. 2006) since the size of the area injured by the ischemia was significantly larger in GDF15 deficient mice. Recently, a similar protective effect of GDF15 regarding ischemia and reperfusion injury in acute kidney disease was proposed (Liu et al. 2020). These conflicting data on GDF15 are indeed intriguing. Possibly, GDF15 exerts its tissue protective effects, at least partly, from signaling through the phosphoinositide 3-OH kinase-Akt pathway. In conclusion, the existing and somewhat diverging data on GDF15 and CVD versus ischemia reperfusion injury, suggest that GDF15 might have different biological effects depending on the environment. Clearly, the role of GDF15 in inflammation and cancer, including CLL, and as prognostic biomarker in CVD and cancer warrants further investigations.

In line with prior data on cancer and myostatin levels, we present novel data on significantly higher levels of myostatin in plasma of CLL patients. Myostatin, also known as GDF8, is another member of the TGFβ superfamily. It has previously been

shown to act as a negative regulator of skeletal muscle mass in an animal model (McPherron, Lawler, and Lee 1997). Elevated levels of myostatin have been detected in human cancer as well as in experimental cancer cachexia (Costelli et al. 2008). Myostatin mediates atherosclerotic progress in the abdominal aorta (Verzola et al. 2017) and in a recent study in patients with heart failure, higher serum levels of myostatin were found in patients compared to healthy controls. Moreover, in the same study, elevated myostatin levels correlated with the severity of the heart failure and prognosis (Chen et al. 2019). Previous data on myostatin and CLL are absent and its role in CLL needs further exploration.

Galectin-3 is an inflammatory marker, and its expression has also been linked to disease progression in chronic myeloid leukemia and multiple myeloma. Overall, data on galectin-3 and CLL are scarce. One study using peripheral blood (PB) from 85 CLL cases and 12 controls showed downregulation of galectin-3 in CLL cells, especially in cases with progressive disease (Asgarian-Omran et al. 2010). However, a more recent study including PB and BM from 67 CLL patients, published in 2019 (Michalová et al. 2019) found an increased expression of intracellular galectin-3 in CLL cells, especially in those with deletion 17p. In line with the latter study, we observed high levels of galectin-3 in plasma in patients with mainly indolent CLL cases. Obviously, we cannot directly compare our results with those in the two previous studies, since they measured intracellular galectin-3 content. Regarding its association with CVD, a study from 2012 showed elevated levels of galectin-3 in serum/plasma, which strongly correlated to higher age and several risk factors for CVD in a population-based cohort (de Boer et al. 2012). More recently, it has been found in higher concentrations in patients with atrial fibrillation (Gong et al. 2020). This might be of special interest regarding CLL patients in need of BTKi treatment, who are at risk of AF, in helping clinicians to identify CLL patients at certain risk for developing AF.

It has previously been shown that MMP9 is expressed on the cell surface of CLL cells (Kamiguti et al. 2004). The same study revealed that high levels of intracellular MMP9 in CLL cells correlated with advanced clinical stage. An *in vitro* study from 2019, in which CLL cells were cultured in MMP9-containing medium, showed an upregulation of pro-angiogenic genes such as VEGF (Aguilera-Montilla et al. 2019). In line with previous studies, we found higher levels of MMP9 in plasma from our CLL patients. MMP9's role in acute cardiovascular syndrome has been studied, and high serum MMP9 correlated to poor cardiovascular outcome, including survival (Lahdentausta et al. 2018).

TNFα is known to promote inflammation through its receptors TNFαR1 and R2, expressed in both plasma membrane bound and soluble (s) forms. In 1992, Waage et al. reported increased expression of these two receptors on CLL cells and elevated levels of the soluble receptors in serum (Waage et al. 1992). More recently, in 2018, PB from 247 German CLL patients included in the CLL 8 trial was analyzed for sTNFR1 in serum, finding higher levels in CLL patients compared with controls, in line with our results. Of interest, elevated serum levels of sTNFR1 correlated (multivariate analysis) with shorter overall survival and appeared to be a prognostic marker for overall survival and tumor-associated death (Dürr et al. 2018).

Cardiovascular disease and its association with elevated levels of sTNFR1 and R2 has been reported in a Swedish case–control study including patients recruited from three large epidemiological projects. The investigators reported high levels of sTNFR1 and sTNFR2 in combination with elevated CRP, especially in women, correlated with an increased risk of myocardial infarction (Carlsson et al. 2018).

Wada et al. Wada et al. (2010) demonstrated in 2010 that the plasma levels of sVEGFr2 were increased in individuals with metabolic syndrome. Furthermore, the levels of sVEGFr2 correlated positively and significantly with several known risk factors for CVD, such as fasting plasma glucose, diastolic blood pressure and high sensitive CRP. The same study included analyses of circulating VEGF and sVEGFr1, however, their levels did not correlate to metabolic syndrome. There are some previous data on CLL and VEGF. Among these, in a study from 2005, the concentrations of VEGF, sVEGFR1 and 2 in serum from 83 treatment naïve CLL patients and 20 controls were analyzed. The authors reported higher concentrations of VEGF and sVEGFR2 in more advanced stages of CLL patients, whereas sVEGFR1 did not correlate to disease stage (Gora-Tybor, Blonski, and Robak 2005). We have not performed analyses to correlate our results to disease stage since our cohort entails only a few cases of Binet B and C stage. Nevertheless, significantly higher concentration of sVEGFR2 in plasma was seen in our population of mostly less advanced CLL patients compared with controls. Interestingly, in 2015, a possible association

to develop CLL and a certain genotype of sVEGFR2 (rs2010963) was observed (Góra-Tybor et al. 2015).

CRP, as well as PTX3, belongs to the pentraxin protein superfamily. In contrast to the hepatic protein CRP, PTX3 is produced by different cell types and has been found to play a role in tissue repair (Bottazzi et al. 2010; Doni et al. 2019). Furthermore, PTX3 is produced by endothelial and vascular smooth muscle cells in response to inflammation, and its expression correlates with the severity of coronary heart disease (Liu et al. 2015). In previous studies, both CRP and PTX3 are suggested biomarkers of CVD. Due to this, in combination with the absence of data on PTX3 and CLL, we found it relevant to enclose the biomarker in our study. However, neither CRP nor PTX3, displayed a significant difference in plasma levels between CLL patients and controls. This is somewhat surprising results since high sensitive CRP is a well-established CVD risk biomarker (Yousuf et al. 2013). Thus, a newly published Chinese study from 2021 has shown that the CRP/albumin ratio might be a useful tool to predict overall survival in newly diagnosed CLL patients (Tang et al. 2021). This study demonstrated that CLL patients with higher CRP/albumin ratio, corresponding to elevated CRP and/or lower albumin levels, reflecting inflammation and/or malnutrition, had shorter OS. However, in this CLL cohort of 322 patients, the majority (68.9%) presented with Binet B or C ($n = 222$) whereas only 31.1% ($n = 100$) were classified as Binet A. Additionally, no matched controls were enrolled in the study. Possibly, the lack of significant difference in CRP between CLL patients and controls in our cohort is due to the fact the majority of patients had indolent disease (Binet A) and that CRP in this regard is too unspecific. Exclusive analyzes of low-grade CRP elevations, by using high sensitive CRP, might be of interest in future studies entailing CLL patients with both indolent and advanced disease as well as controls.

Several biomarkers have been studied in CLL, but currently B2M is the only one used in clinical practice as a marker of high tumor burden, advanced disease stage, and poor prognosis. B2M is included in the most recent clinical staging system. ("An international prognostic index for patients with chronic lymphocytic leukemia (CLL-IPI): a meta-analysis of individual patient data" 2016; Condoluci et al. 2020). Regarding its role in CVD, B2M has been suggested as a biomarker for coronary heart disease (You et al. 2017). In this study, we included B2M partly as a positive control biomarker, i.e. due to its role as a negative prognostic biomarker in CLL. Not surprisingly, B2M differed significantly between CLL patients and controls.

Strengths of our study are the population-based cohort of untreated CLL patients, reflecting a real-world CLL population, and the use of healthy age- and sex-matched controls. Considering the heterogeneity of CLL, a limitation is the relatively small cohort of CLL patients, especially when it comes to associating disease stage with biomarker concentrations. In our CLL cohort, the vast majority (82%) was indolent, correlating to Binet A (Binet A $n = 104$, Binet B $n = 13$, Binet C $n = 10$, unknown $n = 12$), thus, differences in biomarker concentration in relation to CLL stage are statistically troublesome to analyze and draw conclusions from. Yet, in this indolent cohort of CLL patients, there is an obvious diverging pattern of CVD biomarkers compared with controls. Clinically, these CLL patients rarely present with any symptoms of their CLL disease but still have diverging blood levels of several biomarkers also associated with CVD. With these results and this background, it is obvious that large population-based studies are warranted. The Uppsala U-CAN CLL cohort is continuously including CLL patients, as well as follow up samples, enabling future analyses, which is of interest with regard to potential modulation of CVD biomarker expression in response to CLL therapy.

In summary, using a population-based cohort of untreated CLL patients and comparing them with healthy controls, we have shown significantly diverging levels of 8 out of 11 different biomarkers associated with CVD and inflammation, including the novel findings of increased GDF15 and myostatin expression. Further prospective studies in patients with neoplastic diseases should investigate the more exact association between these biomarkers and the development of CVD. In CLL, their correlation with response to BTKis and other CLL-specific treatments are of particular interest.

Author contributions

A.L. and T.K. suggested the chosen biomarkers. K.L. provided the clinical data of the CLL patients from the Uppsala U-CAN CLL biobank patients, and A.L. the controls. M.T. performed the statistical analyses. K.L. wrote the manuscript together with T.K. after discussing the data and results with A.L. and M.H. All authors analytically revised the manuscript and gave final approval for submission.

Disclosure statement

None of the authors have any conflict of interest to declare.

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