CHAPTER 1

Introduction and review of circulating biomarkers in Hodgkin Lymphoma

Modified from: Circulating biomarkers in classical Hodgkin Lymphoma

Wouter J. Plattel, Anke van den Berg, Lydia Visser, Hanneke C. Kluin-Nelemans, Gustaaf W. van Imhoff, and Arjan Diepstra

In preparation
Introduction and scope of the thesis

Epidemiology and pathobiology
Hodgkin lymphoma (HL), formally known as Hodgkin’s disease, is a malignant disease named after Thomas Hodgkin who first described this entity in 1832. HL has an age-standardized incidence rate of 3 per 100,000 per year and accounts for 15-25% of all lymphoma patients in the western world. Remarkably, about half of the patients are adolescents or young adults.

HL is characterized by the presence of large malignant cells, which contain two nuclei or nuclear lobes. Together with its mononuclear variants, these cells are called Hodgkin Reed-Sternberg (HRS) cells and are typical for classical HL (cHL). Classical HL accounts for approximately 95% of all HL cases and the remaining 5% consist of the non-classical subtype nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). This non-classical variant is regarded as a distinct entity based on its distinct histopathological features, clinical presentation and clinical behavior. In this thesis we will focus on the cHL subtype only.

Classical HL has a unique and fascinating pathobiology. First, the malignant cells are greatly outnumbered by a massive inflammatory component which is unique not only among lymphomas but also among solid cancers (Figure 1). Second, HRS are of B-cell origin, but they have lost their entire B-cell phenotype including surface immunoglobulin expression. Last, about 30% of cHL cases contain the Epstein Barr Virus (EBV), which is involved in the pathogenesis of these cases.

Figure 1. Histopathological pictures of cHL. The scarcity of the HRS cells with its surrounding microenvironment (A) and an example of the interaction between HRS cells and its microenvironment as demonstrated by HRS cell specific TARC staining (blue) and its receptor CCR4 (brown) on lymphocytes (B). H = Hodgkin cells; RS = Reed Sternberg cells; T = T-cells; H = histiocytes; E = eosinophils; P = plasma cells.
Normal immature B-cells undergo somatic recombination in germinal centers to transform into plasma cells or memory B-cells containing a B-cell receptor (BCR) with high affinity to a certain antigen. B-cells that lack a high affinity BCR or express a BCR with high affinity to self-antigens are either clonally deleted, undergo receptor editing or end in an anergic state. The lack of a BCR or a functional BCR as characteristic for HRS cells would normally lead to apoptosis. However, HRS cells have found a way to escape from apoptosis during the germinal center reaction. Constitutive activation of the canonical and non-canonical nuclear factor kappa-B (NFkB) pathways are a main survival mechanism by upregulating anti-apoptotic genes like c-FLIP and XIAP. The canonical NFkB signaling can be activated via tumor necrosis receptor (TNFR) family members like CD30, CD40, RANK and TNFR1 that are expressed on the cell membrane of HRS cells. The non-canonical pathway can be activated by both autocrine and exocrine signaling by HRS cells and surrounding immune cells, respectively. Latent membrane protein 1 (LMP-1) is an EBV-derived protein that mimics CD40 receptor stimulation, whereas LMP2 mimics BCR signaling. Together these signals can also induce activation of the NFkB pathway in EBV+ cHL. The extensive micro-environment of cHL consists of varying numbers of plasma cells, granulocytes, histiocytes, eosinophils, mast cells and macrophages, but the main cell type present in this reactive infiltrate is the T-cell (Figure 2). Remarkably, this reactive infiltrate is unable to induce an effective immune response against the HRS cells. Moreover, the HRS cells need the reactive infiltrate to survive. This protective micro-environment is for a main part self-orchestrated by the HRS cells by various mechanisms. Constitutive activation of the NFkB and Janus kinase–signal transducer and activator of transcription (JAK-STAT) signaling pathways are central players in this aberrant interaction. Genetic alterations including copy number gains and activating mutations have been found in components of both the NFkB and JAK-STAT pathways in 53% and 90% of cases, respectively. Moreover, HRS cells directly suppress a Th1 response by producing IL-10 and TGF-β and by expressing cell surface molecules including Fas ligand and galectin-1 that are associated with decreased amounts of effector T-cells surrounding the HRS cells. On the other hand, HRS cells promote and attract T-helper 2 cells (Th2) and regulatory T-cells (Treg) type immune cells by secreting CCL17 (also known as thymus and activation regulated chemokine, TARC), CCL5, CCL22 chemokines and IL-7, the latter of which enables differentiation of naïve CD4+ T-cells into Treg cells. The T-cells in the reactive infiltrate mainly consist of CD4+ T-cells and they produce Th2 stimulatory cytokines like IL-4 and IL-5 and IL-10. The inhibition of an effective Th1 response is illustrated by the scarcity of CD8+ cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells in the direct vicinity of the tumor cells. In addition, the CD4+ T-cells directly surrounding the HRS cells seem to be in an anergic state as a result of several mechanisms including lack of expression of the T-cell activation marker CD26 and variable expression of immune check-point molecules CTLA-4 and PD-1 in these T-cells, as well as increased PD-1 ligand expression by HRS cells and the abundant production of IL-10 and TGF-β.
The composition of the reactive infiltrate and stroma is the basis of a further histopathological separation of cHL into four subtypes: nodular sclerosis cHL, mixed cellularity cHL, lymphocyte rich cHL and lymphocyte depleted cHL. Nodular sclerosis cHL is the most common subtype and represents about 60% of the cHL cases in the western world, whereas mixed cellularity is more common in developing countries and is frequently associated with presence of EBV (about 70%). Although these subtypes have shown differences in gene-expression, cytokine production and also clinical behavior, this distinction does not result in different treatment approaches.

![Cellular composition of cHL and crosstalk between HRS cells and its micro-environment. HRS cell = Hodgkin Reed-Sternberg cell; T = T-cell; NK = Natural killer cell; Treg = regulatory T-cell; Th1 = T-helper 1 cell; plasma = plasma cell.](image-url)

**Staging, treatment and prognostic factors**

After the introduction of multi-agent chemotherapy regimens in the 1960s and improvements in radiotherapy, cHL can now be cured in more than 80% of all newly diagnosed patients (stage I-IV). Current treatment algorithms are based on a combination of clinical and laboratory factors. The Ann Arbor staging system separates early stage from advanced stage patients based on number and location of the involved lymph nodes and presence of extranodal disease. Several large study groups further classify early stage patients for allocation of treatment into...
early favorable and early unfavorable risk groups using slightly different criteria (Table 1). For advanced stage disease, a more widely accepted scoring system, the International Prognostic Score (IPS), has been developed based on six factors with independent significance for freedom of progression of disease in multivariate analysis. However, the clinically utility of the IPS to allocate treatment is limited.

Table 1. Clinical prognostic risk classification for early stage cHL

<table>
<thead>
<tr>
<th></th>
<th>EORTC&lt;sup&gt;a&lt;/sup&gt; favorable</th>
<th>GHSG&lt;sup&gt;b&lt;/sup&gt; favorable</th>
<th>NCCN&lt;sup&gt;c&lt;/sup&gt; favorable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&lt; 50</td>
<td>Not included</td>
<td>Not included</td>
</tr>
<tr>
<td>ESR and B-symptoms</td>
<td>&lt; 50 mm/hr no</td>
<td>&lt; 50 mm/hr no</td>
<td>&lt; 50 mm/hr no</td>
</tr>
<tr>
<td>B-symptoms or</td>
<td>B-symptoms or</td>
<td>B-symptoms</td>
<td>B-symptoms</td>
</tr>
<tr>
<td>&lt; 30 mm/hr +</td>
<td>&lt; 30 mm/hr +</td>
<td>&lt; 30 mm/hr +</td>
<td>&lt; 30 mm/hr +</td>
</tr>
<tr>
<td>B-symptoms</td>
<td>B-symptoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large mediastinal mass</td>
<td>Absent</td>
<td>Absent (advanced stage if present)</td>
<td>Absent</td>
</tr>
<tr>
<td>No. involved sites</td>
<td>&lt; 4</td>
<td>&lt; 3</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>Extranodal disease</td>
<td>Not included</td>
<td>Absent</td>
<td>Not included</td>
</tr>
</tbody>
</table>

<sup>a</sup>European Organisation of Research and Treatment of Cancer; <sup>b</sup>German Hodgkin Study Group; <sup>c</sup>National Comprehensive Cancer Network

With current treatment regimens over 90% of early stage patients can be cured with the combination of 2-4 courses of chemotherapy (Adriamycin, Bleomycin, Vinblastine and Dacarbazine (ABVD)) followed by involved node radiotherapy. The drawbacks of this highly curative combined modality treatment are the long term cardiovascular toxicities and secondary malignancies that can for an important part be attributed to former extensive radiotherapy fields and anthracycline-based therapies. Advanced stage patients can be treated with 6 cycles of ABVD chemotherapy or 4-6 cycles of dose intensified chemotherapy (Bleomycin, Etoposide, Adriamycin, Cyclophosphamide, Oncovin, Procarbazine, Prednisone (escalated BEACOPP)) followed by radiotherapy on residual active disease sites. With these treatments about 75 and 90% of advanced stage patients can be cured, respectively. Especially for patients treated with regimens such as escBEACOPP, the risk of death due to short and long-term treatment-related toxicities is approaching the risk of dying of cHL itself. EscBEACOPP is also strongly associated with infertility in both men and women.

The main challenge in cHL management is therefore to minimize both overtreatment and undertreatment by better tailoring treatment to the prognosis of the individual patient. Recognition of patients at high risk of treatment failure before or early during treatment is therefore of utmost importance.
**FDG-PET guided treatment**

Functional imaging with Fluorodeoxyglucose Positron Emission Tomography (FDG-PET)-scans combined with computer tomography (FDG-PET/CT) has become the standard tool for staging and response assessment in cHL. FDG-PET/CT is more sensitive than conventional imaging and led to significant upstaging in cHL.\textsuperscript{24,25} Staging in the era of FDG-PET/CT has also made routine bone marrow biopsy unnecessary because of its higher sensitivity for bone marrow involvement.\textsuperscript{26,27}

Response evaluation during treatment using FDG-PET scanning (interim FDG-PET (iPET)) enables evaluation of early metabolic changes rather than the morphologic changes visualized by CT occurring later during and after completion of treatment. Several studies using iPET after two or three cycles of ABVD have shown that early metabolic changes are predictive of the final treatment response and PFS.\textsuperscript{28-30} Based on these studies, which were mainly performed among advanced stage patients, several cooperative groups have incorporated iPET imaging in their trials to reduce treatment exposure in responding patients to prevent overtreatment and/or to intensify treatment in case of non-responsiveness. Very recently this strategy of treatment escalation or de-escalation based on iPET has shown to improve progression free survival or safely decrease treatment exposure in early- as well as advanced stage patients.\textsuperscript{17,18,31,32}

Nevertheless, iPET does not perfectly predict final outcome. In patients with a negative iPET after two cycles of ABVD, failure rates were observed ranging from 10% up to 25% in early- and advanced stage patients, respectively.\textsuperscript{17,33} On the other hand, 25% of advanced stage patients treated with ABVD who are iPET positive become FDG-PET negative after completion of ABVD treatment and experience durable remissions.\textsuperscript{34} Also, advanced stage patients treated with escBEACOPP show an excellent prognosis after completion of treatment despite iPET positivity.\textsuperscript{32} Although current efforts are being made to improve iPET adapted strategies by investigating different timing and interpretation methods, there is still a need for better early stratification of high and low risk patients.

**Biomarkers**

A plethora of biomarkers derived from patient tissue or from peripheral blood have shown to correlate with prognosis and survival in cHL. These can be grouped into two categories, one being general factors related to inflammation/ the abundant reactive infiltrate; the second being tumor cell-specific biomarkers. In addition, the biomarkers can be grouped based on being tissue-based or blood-based markers. At this moment, only the blood-based factors such as elevated erythrocyte sedimentation rate, anemia, leukocytosis, lymphocytopenia and hypoalbuminuria are included in clinical prognostic models. Excellent reviews on prognostic factors in cHL have been published during the last years, mainly focusing on tissue markers.\textsuperscript{35-38} A disadvantage of almost all published tissue markers is the lack of reproducibility due to differences in tissue fixation, staining, often difficult scoring methods and impractical cut-offs.
Tumor cell specific biomarkers that correlate with treatment response have the potential to be used in the same way as FDG-PET imaging with additional benefits of low-costs, being more patient-friendly, and potentially more specific. Blood-based biomarkers have the advantage over tissue markers that cell numbers, protein levels or DNA copy numbers can usually be quantified with higher reproducibility using standardized assays. From a clinical view, blood-based biomarkers have the great advantage that they can be sampled not only before but also during and after treatment. During the last 40 years an extensive number of studies have been published on blood-based biomarkers either focusing on prognosis (progression or disease free survival) or on evaluating treatment response. In the remainder of this chapter we summarize both prognostic blood-based biomarkers identified at diagnosis and blood-based biomarkers that can be used as treatment response biomarkers.

**Circulating biomarkers**

**Blood cell counts and systemic inflammatory markers**

**Leukocytes: leukocytosis, lymphocytopenia, monocytosis**

**Leukocytosis** defined as leukocyte count $>15\times10^3/\text{mm}^3$ is a common feature in cHL patients with an incidence of about 10-20% at diagnosis (see Table 2).\textsuperscript{15,39} It is included in the IPS as an independent prognostic factor, but it is not consistently observed as such in recent, albeit smaller studies.\textsuperscript{40,41} Leukocytosis was shown to have prognostic value when other prognostic factors including leukocyte subsets were not taken into account.\textsuperscript{42,43}

**Lymphocytopenia** was already identified in 1971 as an adverse prognostic factor in cHL.\textsuperscript{44} This finding was later confirmed in multiple studies. The Hasenclever study incorporated it into the IPS.\textsuperscript{15,45} Lymphocytopenia, either absolute ($<0.6\times10^9/\text{mm}^3$) or relative ($<8\%$ of total WBC) is observed in about one fifth of the patients at diagnosis. It is hypothesized that lymphocytopenia reflects impaired host immune homeostasis or even depletion of lymphocytes by infiltration into the tumor.\textsuperscript{39,46}

**Monocytosis** was more recently recognized as a negative prognostic factor in cHL.\textsuperscript{39} It has been hypothesized that monocyte counts might reflect number of tumor-associated macrophages, which originate from monocytes.\textsuperscript{39} A monocyte count of $>0.9\times10^9/\text{mm}^3$ was found to correlate with adverse progression-free, disease-free and overall survival. The lymphocyte/monocyte ratio further increased the prognostic impact of monocyte counts. A ratio of $<1.1$ correlated with very poor prognosis both in early and advanced stage patients and was independent of IPS. The independent prognostic value of this ratio was confirmed for overall survival in a non-Western cohort, for both progression free and overall survival in a very large study involving patients from Italy and Israel and in multivariate analysis with interim FDG-PET result.\textsuperscript{41,47,48} In conclusion, there is consistent evidence for a prognostic role of the lymphocyte/monocyte ratio in cHL.
Hemoglobin level and iron metabolism.

**Low Hemoglobin** level or anemia has been identified as a negative prognostic factor in many malignancies, including HL. A hemoglobin level below 10.5 g/dl in both males and females is included in the IPS as a negative prognostic factor, which is present in about 40% of cHL patients at diagnosis. Anemia as seen in cHL patients mirrors anemia of patients with chronic diseases and correlates with interleukin (IL)-6 and hepcidin levels. IL-6 can induce hepcidin, which in turn inhibits release of iron stores from the mononuclear phagocyte system and from the intestine and results in elevated levels of ferritin. Both IL-6 and ferritin correlate with prognosis as has been shown decades ago for ferritin and has been confirmed in multiple recent studies. Clinically, ferritin is mainly used as a reflection of actual iron stores, but ferritin also acts as an acute phase protein. Indeed, ferritin levels highly correlate with inflammatory parameters like erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). In cHL patients, anemia probably reflects an active immune state and impaired iron metabolism. It is currently not known whether anemia, serum IL-6 or ferritin is the most potent prognostic factor or whether they have independent prognostic value since a direct comparison of those markers is lacking.

**Other circulating cells**

In a search for peripheral blood biomarkers that reflect tumor micro-environment or host immune response, there are two smaller studies that have found new prognostic markers. The first study showed that higher levels of circulating **CD34+ myeloid-derived suppressor cells** (immature MDSCs) correlate with adverse prognosis. Multivariate analysis in this iPET treatment adapted cohort, revealed that elevation of CD34+ MDSCs was the only remaining significant parameter for survival and outperformed iPET. Another study found the **CD4/CD19 ratio** to be a negative prognostic factor independent of iPET and stage.

**Systemic inflammatory markers**

Systemic inflammatory markers are detected in about half of the patients with cHL and correlate with tumor burden. These markers mainly reflect the abundant micro-environment characteristic of cHL. The most well-known non-specific inflammatory biomarker is the **ESR**. Elevated ESR (>30 or >50 mm) is present in about half of the patients and is included as one of the risk factors for unfavorable early stage disease by major study groups, i.e., the European Organisation for Research and Treatment of Cancer (EORTC), the German Hodgkin Study Group (GHSG) and the National Comprehensive Cancer Network (NCCN) (see Table 1). The initial finding of a correlation of ESR with clinical outcome in both untreated and treated patients published more than four decades ago was confirmed in subsequent papers. In a more recent paper the ESR remained an important factor in currently applied early stage risk classification. However, the prognostic value of ESR is only modest or even absent in multivariate analysis with other prognostic biomarkers. This can probably be explained by the fact that the ESR is influenced by many other prognostic factors like erythrocyte count, fibrinogen levels, presence of acute phase proteins or increased...
Gamma globulins. The studies investigating these individual factors as well as C-reactive protein are generally small or show correlation only in univariate analysis.59-61

**Low albumin** is a negative prognostic factor in cHL. Albumin is the most abundant plasma protein and accounts for about 15% of the protein producing capacity of the liver. Albumin levels inversely correlate with inflammatory status and inflammatory proteins.62 This can be explained by inhibition of albumin synthesis by molecules associated with inflammatory states like tumor necrosis factor (TNF), IL-11 and IL-6 that shift the protein production of the liver to production of acute phase proteins.63 Low albumin was the only negative prognostic factor in a large cohort of cHL patients treated from 1970 until 1980 in a model with ESR, hemoglobin, alkaline phosphatase and lactate dehydrogenase.57 This international study confirmed the prognostic impact of low albumin levels with a cut off at 4.0 g/dl and was included in the IPS as a negative prognostic factor.15

<table>
<thead>
<tr>
<th>% at diagnosis</th>
<th>HR for PFS</th>
<th>RR (from ref 15)</th>
<th>cut off level</th>
<th>Status#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>15-30</td>
<td>1.35</td>
<td>&lt;10.5 g/dl</td>
<td>Established15,45</td>
</tr>
<tr>
<td>Leukocytosis</td>
<td>15-20</td>
<td>1.41</td>
<td>&gt;15x10⁹/mm³</td>
<td>Established 15,39</td>
</tr>
<tr>
<td>Lymphopenia (L)</td>
<td>15-25</td>
<td>1.38</td>
<td>&lt;600/mm³ or &lt;8%</td>
<td>Established15,45</td>
</tr>
<tr>
<td>Monocytosis (M)</td>
<td>35</td>
<td>1.8</td>
<td>&gt;700-900 cells/ul</td>
<td>Potential39</td>
</tr>
<tr>
<td>L/M ratio</td>
<td>~35</td>
<td>2.9 - 3.8</td>
<td>&lt;1.1 / &lt;2.1</td>
<td>High potential 41,47,48</td>
</tr>
<tr>
<td>ESR</td>
<td>35-50</td>
<td>1.5-1.6*</td>
<td>30-50 mm/1sthr</td>
<td>Established 54-58</td>
</tr>
<tr>
<td>Albumin</td>
<td>40-60</td>
<td>1.49</td>
<td>4 g/dl</td>
<td>Established 15,57</td>
</tr>
<tr>
<td>Ferritin</td>
<td>30-40</td>
<td>4.0</td>
<td>350 ug/l</td>
<td>Potential40,49-53</td>
</tr>
</tbody>
</table>

HR = hazard ratio; PFS = progression free survival; RR = relative risk
* Odds ratio for treatment failure within 2.5 years after diagnosis in early stage patients only
# Definitions used for status: established = biomarkers included in currently applied prognostic models; potential = one or limited number of papers indicating prognostic value; high potential = multiple reports indicating high prognostic impact

**Tumor and microenvironment-derived markers**

**Cytokines, chemokines and soluble receptors.**
Multiple studies addressed the prognostic value of soluble levels of cytokines, chemokines and their receptors. **IL-6** is a pro-inflammatory cytokine produced by HRS cells and by cells from the microenvironment including lymphocytes, macrophages and fibroblasts (see Figure 2). IL-6 levels are elevated in about 20-30% of the patients depending on the applied cut-off.52,62 The prognostic value of IL-6 was confirmed in a study including 30 cytokines, whereas in another study with a case-control design, IL-6 did not have independent prognostic value, showing that thus far the value of IL-6 is inconclusive.52,64
IL-10 is produced by HRS cells and regulatory T-cells and inhibits a Th1 antitumor response. IL-10 levels are elevated in about 40-60% of patients and correlate positively with tumor cell EBV status. The prognostic value of IL-10 is controversial with multiple studies showing a prognostic value of IL-10 independent from clinical parameters, whereas other studies did not find any independent prognostic value. An explanation for these findings might be that in the latter studies multiple cytokines have been included.

The soluble form of the alpha chain of the receptor of IL-2 (sIL-2R, sCD25) is another immunomodulatory molecule that correlates with prognosis in cHL. sCD25 is derived by proteolytic cleavage of CD25 by Matrix metallopeptidase 9 (MMP9) produced by tumor-associated macrophages. Levels of sCD25 correlate with the presence of tumor-associated macrophages in the microenvironment of NHL. sCD25 is a marker for activated B- and T-cells and is considered to be a marker of regulatory T-cells. Upon activation by IL-2, CD25 induces FOXP3 expression in CD4+ T-cells thereby promoting a regulatory T-cell phenotype in the tumor microenvironment. A negative prognostic value of high levels of sCD25 was first reported in 1987. These findings were confirmed in several subsequent studies, but could not be confirmed in other studies.

Soluble IL-1 receptor antagonist (sIL-1RA) is produced by various types of immune cells and is elevated in about 35% of cHL patients. IL-1RA competes with type I and type II IL-1R and can partly neutralize the inflammatory effects of IL-1 secreted by HRS cells. Interestingly, secretion of sIL1RA is dependent on IL-6, IL-13 and IL-10 and the prognostic value of these cytokines might be interdependent.

Soluble CD30 (sCD30) is considered to be derived from HRS cells and thus presents a tumor cell-derived marker. CD30 belongs to the TNFR family and can be actively shed from the membrane resulting in sCD30. High sCD30 serum levels were first described as a negative prognostic marker in 1990. This has been confirmed in multiple studies and was shown to be independent of clinical features, or other soluble molecules.

A few attempts have been made to include a set of these prognostic markers in a new prognostic model. Casasnovas et al. found that the combination of IL-6, sIL-1RA and sCD30 in a three cytokine/soluble receptor model had better prognostic value than the IPS. Unfortunately, no multivariate analysis with individual factors of the IPS was shown. Marri et al. performed a multi-cytokine study in which a two-cytokine model containing IL-6 and sCD25 had the best prognostic value. In this study, sCD30 did not maintain prognostic value in multivariate analysis. Unfortunately, sCD25 was not analyzed in the study by Casasnovas.
In the last decade, **Thymus and Activation Regulated Chemokine (TARC)** or CCL17 has gained interest as a prognostic marker and treatment response marker. TARC is produced by HRS cells at very high levels and is responsible for the attraction of CCR4 positive cells, which are mainly regulatory T-cells and Th2 cells (Figures 1 and 2).\(^6\) Thus, TARC is for an important part responsible for the immunosuppressive direct micro-environment of the HRS cells. In line with the high production, extremely high plasma or serum levels have been observed in cHL patients.\(^87,88\) TARC levels are elevated in >85% of patients at diagnosis and high levels correlate with negative prognostic features and higher disease stage.\(^89\) In line with the fact that stage of disease itself is a potent prognostic factor in cHL, high TARC levels at diagnosis correlated with adverse prognosis.\(^90\) This was confirmed in a large study in which a prognostic model that included both TARC and clinical features showed strong prognostic value.\(^58\) However, in a smaller study no correlation with prognosis was found for TARC.\(^52\) Since elevation of TARC is strikingly high compared to healthy controls, TARC has the ideal features to serve as a biomarker for treatment response.

There are several other protein biomarkers like soluble Galectin-1, sCD163, M-CSF, sCD8, sICAM-1, CA125, B-lymphocyte stimulator and polyclonal free light chains that might serve as prognostic biomarkers since they correlate with adverse clinical or disease characteristics. Future studies are needed to elucidate whether these markers have real prognostic value independent of established markers within current treatment era. Soluble Galectin-1 (sGal-1) and soluble CD163 (sCD163) are of special interest since sGal-1 is thought to be derived from tumor cells itself and sCD163 is the soluble form of the M2 macrophage marker CD163. Tumor-associated macrophages are associated with adverse prognosis in tissue of patients with cHL.\(^91,92\)

| Table 3. Cytokines, chemokines and soluble receptors related to prognosis |
|-----------------|-----------------|-----------------|-----------------|
| % patients with marker | cut off level | Adverse prognosis for high/low levels | Status* |
| IL6 | 20-30 | 30 pg/ml | High | Controversial\(^52,64\) |
| IL-10 | 40-60 | 10 pg/ml | High | Controversial\(^52,59,64,66,69\) |
| sIL2R | 60-75 | ~1000 U/ml | High | Controversial\(^59,73-79\) |
| sIL1RA | 35 | 668 pg/ml | High | Potential\(^62\) |
| sCD30 | 40-80 | 20-200 U/ml | High | High potential\(^59,62,68,73,74,78,82-85\) |
| TARC | 70-90 | 500-10,000 pg/ml | High | Controversial\(^52,58,59\) |

* Definitions used for status: controversial = papers showing contradictory results; potential = one or limited number of papers indicating prognostic value; high potential = multiple reports indicating high prognostic impact
Markers of cell or membrane turnover

**Beta-2-microglobulin (B2M)** is a component of the HLA class I complex. Serum levels are thought to reflect membrane turnover and have been correlated with adverse prognosis in several smaller studies with different treatment regimens. Several reports showed an independent negative prognostic value for high serum lactate dehydrogenase (LDH). However, in the International Consortium on Prognostic factors study, LDH levels did not have a significant prognostic value. Also elevated alkaline phosphatase levels have been linked with adverse prognosis in cHL, but did not remain significant in multivariate analyses in the Hasenclever study.

Molecular markers

High levels of circulating **Epstein Barr Virus DNA** (EBV-DNA) were found to closely correlate with tumor EBV-status as detected by EBER in situ hybridization (EBER-ISH) on tissue samples. Patients with EBV positive disease have detectable EBV DNA levels in their circulation in about 65% of cases. An adverse prognostic value of the presence of plasma EBV-DNA independent of IPS was shown in a relatively large cohort of cHL patients. This is in line with the finding that EBV positivity in tumor tissue has an adverse outcome among elderly cHL patients. The subtype of cHL in elderly patients is more often of the mixed cellularity subtype and the HRS cells more often harbor EBV. Unfortunately, the study by Kanakry et al. did not specify the prognostic value of plasma EBV in relation with age. Jones et al. showed that patients with high pre-treatment EBV-DNA copy numbers showed a decrease in copy numbers rapidly after start of treatment, showing its potential to serve as treatment response biomarker among patients with EBV positive disease.

Circulating microRNAs (miRNAs) have also received increasing attention as non-invasive biomarkers for diagnosis or prognosis in cancer. MiRNAs are small non-coding RNAs of about 20-25 nucleotides that regulate expression of protein coding genes at the post-transcriptional level. In cHL, two miRNAs were found to correlate with treatment response. However, differences in miRNAs disappeared when levels were normalized to a cellular small RNA (RNA U6), indicating possible blood cell origin of the miRNAs. A more recent paper isolated extracellular vesicles from plasma and identified several miRNAs that were enriched in plasma of cHL patients compared to healthy controls. A first analysis showed correlation between a decrease of these vesicle-derived miRNAs and a metabolic response upon treatment.

Studies investigating genomic aberrations have always been challenging because of the scarcity of tumor cells in cHL tissue (see Figure 1). Analysis of **circulating tumor DNA** (ctDNA) levels showed to be feasible in cHL and allows non-invasive detection of tumor cell specific genomic aberrations including mutations and copy number alterations (CNAs). Genomic aberrations in cell-free DNA were initially detected in a pregnant woman, who was later diagnosed with
early stage cHL, indicating the presence of ctDNA. Subsequent testing on nine additional cHL patients revealed the presence of 2p and 9p gains, two commonly observed aberrations in tumor cells of cHL, in ctDNA of seven and five of the nine cHL patients, respectively. A larger scale study that was carried out more recently showed that mutations detected in ctDNA mirror the genetics of cHL tumor cells. Due to the small sample size and heterogeneity of the patient cohort no conclusions could be made on any predictive or prognostic value. Sequential samples collected from a small number of patients showed that persistence of high ctDNA levels correlated with treatment failure or relapse. This indicates its potential to serve as a biomarker for treatment response.

In conclusion, there are multiple circulating biomarkers that have potential to be applied at diagnosis and during treatment to optimize decision making upfront and during treatment. Large multicenter studies are needed to define the optimal set of prognostic biomarkers that can be applied at diagnosis given current treatment regimens and the known prognostic value of iFDG-PET imaging. Research on treatment response biomarkers in cHL is more limited, but several biomarkers have high potential to improve on or to be applied next to FDG-PET imaging.

Scope of the thesis

This thesis aims to address the relevance of selected circulating biomarkers. In the first part of this thesis we study the application of TARC as a biomarker for treatment response and compare TARC with galectin-1, sCD163 and sCD30, and interim FDG-PET imaging. In Chapter 2 we studied the correlation of TARC with clinical response in a cohort of 63 patients from the University Medical Center Groningen (UMCG). In Chapter 3 we added three other potential treatment response biomarkers to compare with TARC, i.e. galectin-1, sCD163 and sCD30 in a larger cohort of 103 patients. In Chapter 4 we compared TARC during treatment (interim TARC) with interim FDG-PET imaging for their ability to predict for modified progression-free survival.

In the second part of this thesis we focus on the applicability of circulating microRNAs as biomarkers in cHL. In Chapter 5 we summarize the current knowledge on microRNAs in cHL. In Chapter 6 we address the pre-analytical, analytical, and post-analytical challenges in circulating microRNA studies. Finally, in Chapter 7 we investigated the expression profiles of circulating microRNAs in serum of a cHL patient cohort from Vancouver.

In Chapter 8, all results are summarized and discussed, followed by a general perspective on the future application of biomarkers in cHL.
References

20. Schaapveld M, Aleman BM, van Eggermond AM, et al. Second Cancer Risk Up to 40 Years


36. Diefenbach C and Steidl C. New strategies


72. Yang ZZ, Grote DM, Ziesmer SC, et al. Soluble IL-2Ralpha facilitates IL-2-mediated immune responses and predicts reduced survival
91. Ouyang J, Plutschow A, Pogge von


Introduction and scope of the thesis


