The Role of Inflammation in Cancer and Mortality

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Content

Chapter 1

General introduction and outline of thesis

J. Fest
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Introduction

The immune system is one of the defence mechanisms that protects our body against infectious diseases. It also has the ability to detect and eliminate tumor cells, and therefore also plays a role in the protection against cancer. [1]

The human immune system consists of two parts, the innate and the adaptive part, that work together meticulously. The innate immune system is the first line defence against pathogenic bacterial and viral invasion. It is mainly composed of cells: granulocytes, macrophages, natural killer cells and dendritic cells, which can recognize and kill pathogens. [1] Additionally, they activate the adaptive immune system. The adaptive immune system has a slower response, however, its cells: T-lymphocytes and B-lymphocytes have a specific recognition and are able to selectively target pathogens or damaged cells for elimination. [1]

There is substantial evidence that later in life, the immune system impairs and that as a result of ageing, the body is less able to regulate inflammatory processes. [1,2] These changes are referred to as immunosenescence and are thought to contribute to an increased incidence of morbidity in the elderly population: not only for cancer, but also for other types of disease.[2] It was long thought that the innate immune system was resistant to these changes, but ageing occurs both in the innate and adaptive immune system. [3,4]

In general, the total number of hematopoetic stem cells in the bone marrow is decreased in the elderly, resulting in a decreased proliferation capacity of almost all blood cells. For instance, T-lymphocytes not only decrease in number, they are also less diverse and have a diminished signalling and regulatory capacity in the elderly. [5] Neutrophils are thought to form an exception as both numbers of bone marrow precursors and peripheral blood neutrophils do not change with age. In contrast, their phagocytic abilities and oxidative bursts do decrease, possibly making them less effective. [2]

Additionally, research among healthy individuals has shown that an advanced age is associated with a hyper-inflammatory state, exemplified by an increased presence of inflammatory markers, such as IL-6, TNF-alpha and acute phase proteins such as C-reactive protein (CRP). [6,7] This is referred to as inflammaging.[6] Many of these markers are associated with morbidity and mortality. [8-10] However it is unclear whether these changes of inflammatory mediators are the result of the normal ageing process and a decline of the immune system or whether they are caused by pre-existing conditions and thus can be seen as indicators of underlying or upcoming disease. [11]

With regard to cancer, the relationship with inflammation is well known, however only partially understood as a result of its complex nature. [12-16] One of the theories is that long term inflammation increases the risk of cancer. For instance a *Helicobacter Pylori* infection is associated with an increased risk of gastric cancer, inflammatory bowel disease with colorectal cancer, and tobacco smoke, in addition to being carcinogenic, can induce chronic inflammation that is associated with lung cancer. [17,18] Another theory is that inflammation

may be a secondary systemic inflammatory response to a yet-undetected tumor. [19] Products of inflammatory processes such as biomarkers measured in blood, can be used to study both hypotheses, but are not able to distinguish them.

The role of the immune system in this setting has recently become of greater interest. Neutrophils were traditionally considered as innocent bystanders in the cancer setting. [20] However it has been hypothesized, that neutrophils may be important in tumor initiation, progression, and metastasis. [20,21] Lymphocytes, on the other hand, are thought to have an anti-tumor effect through their ability to specifically target and then kill cancer cells. [22] A deeper insight into the interaction between the immune system and cancer on a systemic level, might help us with the development of new immunotherapeutic agents.

The aim of this thesis was therefore to gain a greater understanding of the role of the immune system in patients with cancer in general and more specifically in those with pancreatic cancer. In order to do so we studied inflammation-related markers in relation to cancer and mortality both in the healthy, ageing population as well as in patients with (pancreatic) cancer. The setting of the studies presented in this thesis is the Rotterdam Study, a population-based prospective cohort study that has been running since 1989 in a sub-urban area of Rotterdam, the Netherlands. [23,24]

Outline of thesis

So far, no conclusive evidence has been found for a causal relation between CRP levels and risk of cancer. [19] Therefore, in **chapter 2** we present an overview of previous studies on the association between the well-known inflammatory marker CRP and the risk of incident cancer in the general population.

Although CRP is probably the most frequently studied inflammatory maker, the white blood cell (WBC) count has also been investigated often. The total WBC count encompasses several cell types, such as granulocytes, lymphocytes and monocytes, which potentially all play a different role in cancer. To simultaneously study the effect of multiple cell types, the neutrophil-to-lymphocyte ratio (NLR) and the systemic immune-inflammation index (SII) were developed. They are composite markers of absolute peripheral neutrophil (N), lymphocyte (L) and in the case of the SII also platelet (P) counts. They are calculated as followed, respectively: NLR = N/L and SII = N/L x P. [25,26]

Since they are relatively novel, little is known about the added clinical value of these markers, and even reference values in the general population are missing. Therefore, we obtained reference values for the SII, NLR and PLR (platelet-to-lymphocyte ratio = P/L) from the Rotterdam Study (**chapter 3**). Furthermore, we addressed whether these markers change with age.

Next, in **chapter 4**, we studied the potential association between the NLR and overall and cause-specific mortality. Furthermore, it is known that in the elderly, inflammatory markers such as CRP and the erythrocyte sedimentation rate (ESR) are elevated. This is considered part of the normal ageing process. [6] As a result, it has been suggested that, in elderly, moderately increased ESR values are not clinically meaningful and can therefore be disregarded. [27] In **chapter 5** we therefore studied the association between the ESR and mortality to verify whether this suggestion could be substantiated with evidence.

The relationship between inflammation and cancer is well-known, yet, it is unsure whether it is the inflammation that leads to cancer, or whether inflammation is a result of a cancer which is already present. One theory is that low-grade, chronic inflammation increases the risk of cancer. Therefore, the objective of **chapter 6** was to investigate whether an increased SII is an indicator for developing cancer in healthy individuals. We hypothesized that when inflammatory cells play a role in the etiology of cancer, individuals with higher levels of inflammation over a longer period of time, as measured by the SII, are at a higher risk to develop cancer.

Alternatively, inflammation may be considered as a consequence, rather than the cause, of cancer. There is plenty evidence for interaction between tumors and the immune system. [14] It is known that more aggressive cancers outmanoeuvre the immune system by evading immune-surveillance or inhibiting activation of the immune system. [14,28] Immunotherapy interfering with this process has shown to be an effective treatment in aggressive cancers like melanoma and lung cancer. [29,30] One of the most aggressive cancers is pancreatic cancer. [31,32] In contrast to the progress made in the treatment of lung cancer and melanoma, little improvement has been made in the treatment of pancreatic cancer. [32] Probably, one of the reasons is that relatively little is known about the interaction between the immune system and pancreatic cancer. Therefore, we were interested to explore the potential changes in the immune system especially in patients with pancreatic cancer.

In the Netherlands, like in many other European countries, pancreatic cancer mortality was found to be systematically higher than the incidence. [33,34]This suggests that there is an underestimation of the reported incidence of pancreatic cancer. Therefore, we first explored the discrepancy between the national incidence and mortality rates in pancreatic cancer in **chapter 7**. We used the Rotterdam Study to establish the incidence rate of pancreatic cancer and its mortality rates. We then linked pancreatic cancer cases to the national cancer registry to get insight into this potential discrepancy between incidence and mortality rates.

Then, in **chapter 8** we studied the role of the SII prior to the diagnosis of pancreatic cancer. It is well recognized that the immune system plays an important role in cancer surveillance and the elimination of tumor cells. [12,13,15] However it also known that pancreatic cancer is capable of misleading the immune system in such a way that it no longer attacks tumor cells, but rather forms a support structure for the cancer. [28,35] Therefore, we investigated whether

there is an impairment of the immune system already prior to the detection of cancer. For the analyses presented in **chapter 8**, we used multiple measurements and evaluated the change in SII levels in the years up to the diagnosis of pancreatic cancer.

In **chapter 9** we aimed to identify new and validate previously found plasma metabolomic biomarkers. It is well known that the development and progression of pancreatic cancer are associated with alterations in the systemic metabolism such as glucose intolerance, accompanied by anorexia and severe weight loss.[32,36] Circulating metabolites have been proposed as a potentially useful screening tool in pancreatic cancer.[37] We set out to replicate previously found metabolomic biomarkers in five large European population cohorts and find additional biomarkers associated with pancreatic cancer.

In the last chapters of this thesis we present a general discussion, summary and conclusion (**chapters 10 and 11**), in which we discuss whether we can provide an answer to the question whether inflammation causes cancer or whether it is a result of the cancer. Furthermore, we discuss several future perspectives of the studied biomarkers in screening on potential cancer and evaluating response to cancer therapy.

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Chapter 2

C-reactive protein and the risk of incident cancer – a meta-analysis of prospective cohort studies.

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Submitted

Abstract

Background: C-reactive protein (CRP) is a sensitive but nonspecific biomarker of systemic inflammation. CRP levels are (moderately) elevated in patients with cancer. Recently, prospective studies have suggested that CRP is also associated with an increased risk of the development of cancer in the general population. However, so far results on the association between CRP and cancer have been inconclusive.

Methods: We performed a review and meta-analysis of prospective, population-based studies that reported on the association between CRP and cancer incidence. Embase, Web of science, Medline, Google Scholar and the Cochrane Library were searched. Summary hazard ratios (HR) were calculated using inverse variance random-effects models.

Results: A total of 72 cohort studies were selected; 30 cohort and 42 case-control studies which were nested in a cohort. There was a significant association between CRP levels and risk of any cancer with an overall HR of 1.11 (95% confidence interval (CI): 1.06 – 1.16). In sub-analyses, there was a significant association between CRP and risk of lung and breast cancer (HRs 1.29 (95% CI: 1.12 – 1.49) and 1.08 (95% CI: 1.01 – 1.14), respectively), but not for CRP and the risk of colorectal or prostate cancer (HRs 1.07 (95% CI: 0.99 – 1.17) and 1.00 (95% CI: 0.93 – 1.09), respectively).

Conclusion: This meta-analysis showed that CRP is a significantly associated with the incidence of all cancers combined. Specifically for incident lung and breast cancer, but not for colorectal and prostate cancer. Whether the relationship between CRP and cancer is causal is still to be determined.

Introduction

In 1863, Rudolf Virchow observed that leukocytes were present in neoplastic lesions and hypothesized that cancer originates at a site of chronic inflammation. [1] However, whether it is the chronic inflammation that leads to the development of a cancer or whether inflammation is the early consequence of a developing but yet undetected malignancy remains a topic of debate. [2] Both theories are probably not mutually exclusive and could be further investigated by studying specific markers of systemic inflammation. C-reactive protein (CRP) is a sensitive but nonspecific biomarker of systemic inflammation. [3] It is an acute-phase protein that is synthesized in the liver as a response to infection, but can also be increased in patients with chronic inflammatory conditions such as diabetes, atherosclerosis, and cardiovascular disease. [3-5]

It is well-known that CRP levels are (moderately) elevated in patients with cancer. [6] However, in these patients reverse causality could also explain the association between CRP and cancer meaning that an elevated CRP is an inflammatory response to the cancer, and thus a consequence rather than a cause. [6,7] More recently, studies have suggested that CRP is not solely a marker of the presence of disease, but that it is also associated with an increased risk of incident cancers during follow-up in the general population. [7,8] Therefore, prospective studies measuring levels of inflammation including CRP at study entry, long before the diagnosis of cancer, might give a more comprehensive insight into the association between CRP and cancer. Any found association could then be a surrogate marker for inflammation that increases the risk of cancer. Although, several prospective studies have been published, so far no conclusive evidence has been provided for a significant association between CRP and cancer. [9]

To elucidate the role of CRP as a risk factor for incident cancers, we performed a review and meta-analysis of prospective cohort studies and nested case-control studies that investigate the association between the inflammatory marker CRP and cancer incidence in the general population.

Methods

Literature search

This systematic review was conducted following the guidelines of the PRISMA statement. [10] In December 2017 and March 2019, Embase, Medline Ovid, Web of Science, Cochrane CENTRAL and Google scholar were searched for epidemiological studies investigating the association between inflammation, as represented by circulating CRP and the subsequent risk of any solid cancer (for search term see **Supplementary Materials**). [11]

Two independent reviewers (JF and RR) manually screened titles and abstracts, and full articles if necessary, of all citations retrieved from the search and checked them for eligibility. Any disagreement was resolved by consensus.

Eligibility criteria

Eligible studies were those that were observational studies with a prospective design [cohort studies or case-control studies which were nested in a cohort], that assessed the association between CRP and the subsequent risk of any solid cancer. We only included epidemiological studies in adults. No randomized controlled trails were available for our research question. We excluded studies that used CRP for adjustment, stratification or as part of a score, without reporting the individual association with CRP. Meta-analyses were not included, but bibliographies of included publications in the meta-analysis were checked for studies that were potentially missed by our search. Finally, we limited inclusions to publications written in English.

Data extraction

From each eligible study, we collected the following information: first author, year of publication, design (nested case-control or cohort), number of cases and controls or population participants, exposure and outcome measured and the maximally adjusted reported effect estimates; odds ratios (OR) for nested cases-control studies and hazard ratios (HR) for cohort studies. The methodological quality of the included studies was assessed by means of the Newcastle-Ottawa scale for non-randomized studies. [12] According to this scale, studies with a score of six out of nine points or above are considered as of high quality.

Statistical analysis

Analyses were performed with Comprehensive Meta-analysis® software version 2 (Biostat, Englewood, New Jersey, USA) and RevMan 5.1 (http://ims.cochrane.org/revman/download).

We pooled those studies that analyzed CRP continuously and that reported the same outcome measure. We performed a meta-analysis for the outcome any cancer and additionally for each of the four major solid cancers: lung, colorectal, breast and prostate cancer. [13]

Pooled effect estimates were reported as HR or OR with 95% confidence intervals. Meta-analyses were conducted using inverse variance random-effects models. [14] Between-study heterogeneity was assessed by means of the I^2 value which measures the percentage of variability in risk effect estimates that is due to between study heterogeneity rather than

chance. [15] Publication bias was assessed from funnel plots in which the log HR for each study was plotted against its standard error. Any symmetry in the plots might suggest a form of publication bias.

Results

A total 5,417 publications were identified in our search in the Embase, Medline Ovid, Web of Science, Cochrane CENTRAL and Google scholar databases (4,944 in the initial search and an additional 473 in the updated search). We found 72 prospective studies that reported on the association between CRP and cancer; 30 cohort studies and 42 nested case-control studies (see **Figure 1, Table 1** and **Table 2**, respectively).

All included studies scored at least six points on the Newcastle-Ottowa scale, for the cohort studies, 19 scored nine out of nine points (63.3%, see **Table 1** and **Table 2**).

There was variation in the primary outcome, most studies chose incidence of all cancers as a main outcome and additionally assessed the four major cancers: lung, colorectal, breast and prostate cancer or either one of these outcomes as a primary outcome. [7,8,16-65] However, several other malignancies were also studied: endometrial [66,67], esophageal [68], gastric [69], liver [70,71], ovarian [55,72-77], pancreatic [78-80], penile [81], testicular [81] and thyroid cancer [82,83] (see **Table 1** and **Table 2**).

There also was a high variation in the reporting of the exposure measures. In some of the studies no high sensitivity CRP measurement was available, only reporting of a CRP ≥ 10.0 mg/L for an analysis. Furthermore, CRP was analyzed in different ways, e.g. continuously, in tertiles, quartiles or quintiles or different cut-off points (see **Table 1** and **Table 2**). As a result there were too few nested case-control studies that reported the same exposure and outcome measure to perform a meta-analysis (see **Table 2**).

Incidence of all cancers

There were nine cohort studies that reported on the incidence of all cancers combined and analysed CRP continuously (ln mg/L). They comprised a total of 38,254 individuals of whom 4,997 developed an incident cancer. There was a significant association, with an increased risk of 11% for each increase in logarithmic [ln] mg/L CRP (HR 1.11; 95% CI: 1.06 – 1.16; see **Figure 2**).

Table 1. Overview of cohort studies that reported on the association between CRP and cancer incidence.

Author	Year	Country	Cohort	Number patients	Cases	Follow-up (years)	Exposure	Outcome	Study Quality
Allin [7]	2009	Denmark	CHS	10,408	1,624	13 (median)	Clinical cut-off	Any cancer; 4 major	8/9
Allin [18]	2016	Denmark	CGPS	84,000	4,081	4.8 (median)	Tertiles	Any cancer; 4 major	8/9
Brasky [89]	2018	USA	WHI	24,205 (F)	Ovarian: 153 Kidney: 110 MM: 137	17.9 (total)	Quartiles	Ovarian, Kidney, Multiple myeoloma	9/9
Busch [19]	2018	USA	WHI	132,262 (F)	6583	5.9 (median)	Continuously	Breast	9/9
Demb [22]	2019		HealthABC	2,323	89	11.9 (median)	Continuously, Tertiles	Lung	8/9
dos Santos Silva [23]	2010	England, Scotland	NPHS-I NPHS-II TPT	NPHS-I: 2,048 (M) NPHS-II: 2,055 (M) TPT: 10,704 (M)	NPHS-I: 442 NPHS-II: 30 TPT: 1439	NPHS-I: 25.6 (median) NPHS-II: 11.7 (median) TPT 12.4 (total)	Continuously, Quartiles	Any cancer; lung, colorectal	8/9
Frydenberg [26]	2016	Norway	Tromsø	8,130 (F)	192	14.6 yrs (total)	Continuously, Tertiles	Breast	9/9
Ghoshal [81]	2017	Sweden	AMORIS	205,717 (M)	Penile: 50 Testicular: 125	20.3 (mean)	Continuously	Penile and testicular	8/9
Ghoshal [83]	2018	Sweden	AMORIS	226,212	202	19.6 (mean)	CRP ≥ 10.0 mg/L	Thyroid	8/9
Ghuman [28]	2017	Sweden	AMORIS	325,599	4,764	18.97 (median)	CRP ≥ 10.0 mg/L	Colorectal	9/9
Gunter [30]	2015	USA	WHI	93,676 (F)	875	-	Quartiles	Breast	8/9
Heikkila [31]	2009	UK	BWHHS Caerphilly	BWHHS: 4,286 (F) Caerphilly: 2,398 (M)	BWHHS: 200 Caerphilly: 247	-	Continuously	Any cancer; 4 major	8/9
Il'yasova [32]	2005	USA	HealthABC	2,438	296	5.5 (average)	Continuously	Any cancer; 4 major	8/9
Izano [34]	2016	USA	HealthABC	2,490	55	Median 11.9 years	Continuously, Tertiles	Colorectal	9/9
Morrison [37]	2016	Finland	KIHD	2,570 (M)	653	26 (average)	Quartiles	Any cancer	9/9
Nelson [39]	2017	USA	WHI	17,841 (F)	1114	13.6 (average)	Continuously	Breast	9/9
Pierce [42]	2009	USA	CHS	2,234 (M)	215	8.7 (mean)	Continuously, Quartiles	Prostate	9/9
Poole [75]	2013	USA	WHS	WHS: 28,345 (F)	159	-	Quartiles	Ovarian	8/9
Prizment [45]	2011	USA	ARIC	9,836	166	17.2 (average)	Quartiles	Colorectal	9/9

Table 1. Continued

Prizment [46]	2013	USA	ARIC	7,603	1,929	-	Continuously	Any cancer; 4 major	9/9
Siemes [8]	2006	Netherlands	RS	7,017	780	10.2 (mean)	Continuously, Clinical cut-off	Any cancer; 4 major	9/9
Toriola [53]	2013	Finland	KIHD	2,571 (M)	203	24 (average)	Tertiles	Prostate	9/9
van Hemelrijck [57]	2011	Sweden	AMORIS	102,749	6,913	-	CRP ≥ 10.0 mg/L	Any cancer	9/9
van Hemelrijck [58]	2011	Sweden	AMORIS	34,891 (M)	1,004	7.5 (mean)	CRP ≥ 10.0 mg/L	Prostate	9/9
Wang [60]	2015	USA	WHS	27,900 (F)	1,919	8.5 (average)	Quintiles	Breast	9/9
Wang [59]	2015	China	CKFC	19, 437 (F)	322	-	CRP ≥ 10.0 mg/L	Any cancer; lung colorectal, breast	7/9
Wulanningshi [62]	2016	Sweden	AMORIS	155,179 (F)	6,606	18.3 (mean)	CRP ≥ 10.0 mg/L	Breast	9/9
Yeung [63]	2013	China	CRISPS	2,893	205	16.0 (median)	Continuously	Any cancer	9/9
Zhang [64]	2005	USA	WHS	27,913 (F)	169	10.8 (total)	Clinical cut-off	Colorectal	9/9
Zhang [65]	2007	USA	WHS	27,919 (F)	892	10 (mean)	Quintiles	Breast	9/9

CHS: Copenhagen City Heart Study, CGPS: Copenhagen General Population Study, WHI: Women's Health Initiative, HealthABC: Health Ageing and Body Composition Study, NPHS: Northwick Park Heart Study, TPT: Thrombosis Prevention Trial, AMORIS: Apolipoprotein-related MOrtality RISk, BWHHS: Brittish Women's Heart and Health Study, WHS: Women's Health Study, ARIC: Atherosclerosis Risk in Communities Study, KIHD: Kupio Ischemic Heart Disease Risk Factor Study, CKFC: Chinese Kailuan Female Cohort, RS: Rotterdam Study

MM: multiple myeloma

Table 2. Overview of nested case-control studies that reported on the association between CRP and cancer incidence.

Author	Year	Country	Cohort	Cases/controls	Exposure	Outcome	Study Quality
Agnoli [16]	2017	Italy	EPIC	351/351	Continuously, tertiles	Breast	7/9
Aleksandrova [17]	2010	Europe	EPIC	Colon: 696/696 Rectal: 400/400	Continuously, quintiles	Colon and rectal	8/9
Aleksandrova [70]	2014	Europe	EPIC	HCC: 125/250 GBTC: 137/274 IBD: 34/68	Continuously, tertiles	HCC, IBD, GBTC	8/9
Bao [78]	2013	USA	HPFS, NHS, PHS, WHI & WHS	470/1,094	Continuously, quintiles	Pancreatic	8/9
Chan [20]	2011	USA	NHS	280/555	Quartiles	Colorectal	8/9
Chaturvedi [21]	2010	USA	PLCO Cancer Screening Trial	592/670	Quartiles	Lung	8/9
Chen [71]	2015	China	LNIT	220/1,018	Continuously, quartiles	Liver	9/9
Cook [68]	2018	USA, Europe	CPS-II, EPIC, MEC, PCPT, PLCO, SBES, WHI	296/296	Quartiles	Oesophageal	9/9
Dossus [66]	2010	Europe	EPIC	305/574	Quartiles	Endometrial	8/9
Dossus [24]	2014	France	E3N prospective cohort	549/1,040	Continously	Breast	7/9
Dossus [82]	2017	Europe	EPIC	475/1,016	Tertiles	Thyroid	8/9
Douglas [79]	2011	Finland, USA	ATBC Study & PLCO	ATBC: 311/510 PLCO: 182/374	Continuously, quintiles	Pancreatic	7/9
Erlinger [25]	2004	USA	CLUE II cohort	172/342	Continuously, quartiles	Colon	9/9
Gaudet [27]	2013	USA	CPS-II	302/302	Tertiles	Breast	8/9
Grote [80]	2012	Europe	EPIC	455/455	Continuously, quartiles	Pancreatic	8/9
Gunter [29]	2006	Finland	HealthABC Study	130/260	Quartiles	Colonrectal	8/9
Ito [33]	2005	Japan	JACC Study	141/327	Tertiles	Colorectal	7/9
Kim [35]	2016	USA	PHS	268/446	Quartiles	Colorectal	7/9
Lee [36]	2011	Korea	-	729/80,052	Clinical cut-off	Any cancer	6/9
Lundin [72]	2009	Sweden, USA, Italy	NSHDS, WHS, ORDET	237/427	Tertiles	Ovarian	7/9
McSorley [73]	2007	USA, UK	CLUE-I, CLUE-II, WCCP, BCDDP, NCIBMP	166/335	Tertiles	Ovarian	8/9
Muller [38]	2019	World wide	Lung Cancer Cohort Consortium	5,299/5,299	Continously	Lung	9/9
Ohishi [90]	2014	Japan	Adult Health Study	224/644	Tertiles	HCC	9/9
Ollberding [40]	2013	USA, Hawaï	MEC	706/706	Quartiles	Breast	9/9

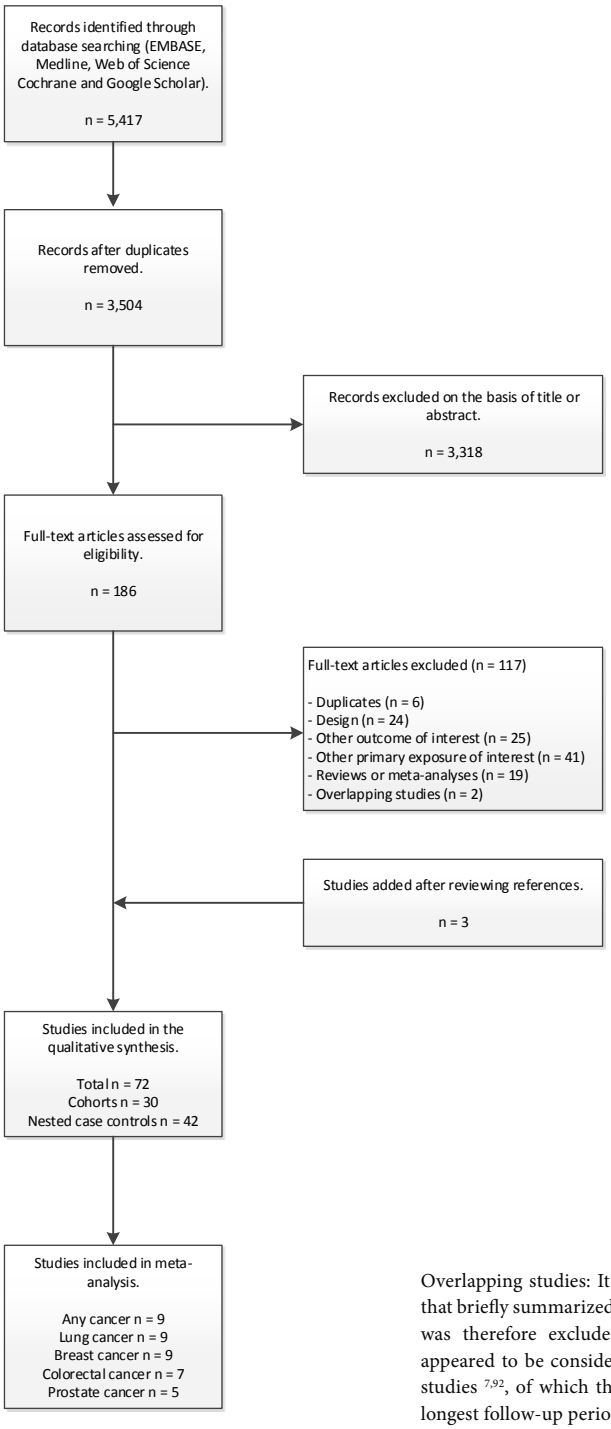
Table 2. Continued

Ose [74]	2015	Europe	EPIC	754/1497	Continuously, tertiles	Ovarian	9/9
Otani [41]	2006	Japan	JPHC Study	375/750	Quartiles	Colorectal	9/9
Pine [43]	2011	USA	PLCO	532/595	High *versus* low	Lung	7/9
Platz [44]	2004	USA	CLUE-II	264/264	Quartiles	Prostate	9/9
Poole [75]	2013	USA	NHS/NHS-II	217/434	Quartiles	Ovarian	8/9
Rifai [47]	2002	USA	WHS	513/513	Quartiles	Any cancer	9/9
Sasazuki [69]	2010	Japan	JPHC Study	494/494	High *versus* low	Gastric	8/9
Shiels [48]	2013	USA	PLCO	526/592	Quartiles	Lung	7/9
Shiels [49]	2017	China	SWHS	248/263	Quartiles	Lung	9/9
Song [50]	2013	USA	HPFS	274/532	Quartiles	Colorectal	8/9
Stark [51]	2009	USA	PHS	602/1,142	Quartiles	Prostate	7/9
Toriola [77]	2011	Finland	FMC	SCST: 58/144 GCT: 30/74	Continuously	Ovarian (non-epithelial)	8/9
Toriola [76]	2011	Finland	FMC	170/170	Tertiles	Ovarian	8/9
Toriola [52]	2013	USA	WHI	953/953	Quintiles	Colorectal	8/9
Touvier [54]	2013	France	SU.VI.MAX	512/1024	Quartiles	Any cancer	8/9
Trabert [55]	2014	USA	PLCO	149/149	Tertiles	Ovarian	8/9
Trichopoulos [56]	2006	Greece	EPIC	496/996	Continuously	Any cancer	8/9
Wang [67]	2011	USA	WHI	151/301	Quartiles	Endometrial	8/9
Wang [60]	2015	USA	NHS	943/1221	Quintiles	Breast	9/9
Wu [61]	2013	China	SMHS	288/576	Tertiles	Colorectal	8/9

EPIC: European Prospective Investigation into Cancer and Nutrition, HPFS: Health Professionals Follow-Up Study, NHS: Nurses' Health Study, PHS: Physicians's Health Study, WHI: Women's Health Initiative, WHS: Women's Health Study, PLCO: Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial, Alpha-Tocopherol, CLUE: "Give us a CLUE to cancer and heart disease", LNIT: Linxian Nutrition Intervention Trials: Dysplasia Trial and the General Population Trial, MEC: Multi-ethnic Cohort, JPHC: Japan Public Health Center-based prospective study, ATBC: Beta-Carotene Cancer Prevention Study, CPS: Cancer Prevention Study, HealthABC: Health Ageing and Body Composition Study, JACC: Japan Collaborative Cohort Study for Evaluation of Cancer Risk, NSHDS: Northern Sweden Health and Disease Study, ORDET: prospective study on hormones, diet, and breast cancer risk, WCCP: Women's Cancer Control Program, BCDDP: Breast Cancer Detection Demonstration Project, NCIBMP: National Cancer Institute's Biological Markers Project, PCPT: Prostate Cancer Prevention Trial, SBES: Study of Baerrett's Esophagus, FMC: The Finnish Mobile Clinic Health Examination Survey, SU.VI.MAX: The Supplementation en Vitamines et Mineraux Antioxydants, SMHS: Shanghai Men's Health Study.

HCC: hepatocellular carcinoma, IBD: intra-hepatic bile duct, GBTC: gallbladder and extra-hepatic bile duct

Figure 1. PRISMA diagram showing selection of articles for review.



Overlapping studies: It appeared there was one study [91] that briefly summarized two other studies [25,44], the former was therefore excluded from the review. There also appeared to be considerable overlap between two other studies [7,92], of which the study with the most cases and longest follow-up period was included in the review [7].

Incidence of four major cancers

Figures 3-6 show the results of de meta-analyses for lung, colorectal, breast and prostate cancer, respectively. The random effects model showed a significant association between CRP and incident lung cancer (HR 1.29; 95% CI: 1.12 – 1.49) and between CRP and breast cancer (HR 1.08; 95% CI: 1.01 – 1.14). No significant associations were found for colorectal and prostate cancer (HR 1.07; 95% CI: 0.99 – 1.17 and HR 1.00; 95% CI: 0.93 – 1.09, respectively).

Effect estimates

There was little to moderate heterogeneity amongst the studies that reported on incident colorectal (I^2 = 12%, P = 0.33), prostate (I^2 = 13%, P = 0.33) or any cancer (I^2 = 39%, P = 0.11). Even though studies largely overlapped, those that reported on lung and breast cancer had showed a high heterogeneity (lung cancer: I^2 = 75%, P < 0.01 and breast cancer: I^2 = 73%, P < 0.01).

Publication bias

In none of the meta-analyses did visual inspection of the funnel plots reveal asymmetry, indicating there was no evidence of publication bias (**Supplementary Figures 1-5**).

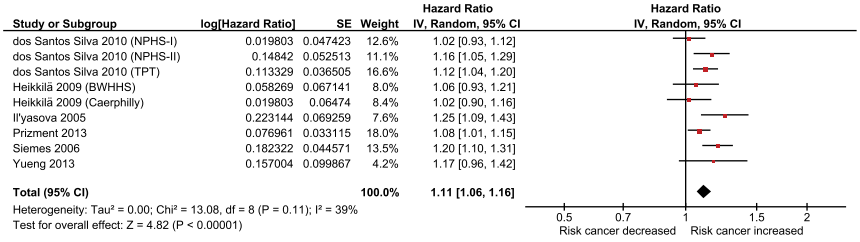


Figure 2. Forrest plot for the association between CRP and any solid cancer.

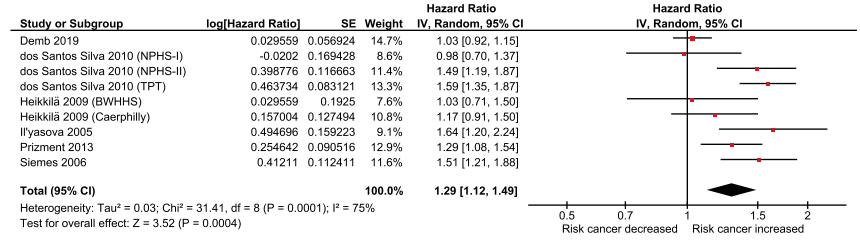


Figure 3. Forrest plot for the association between CRP and lung cancer.

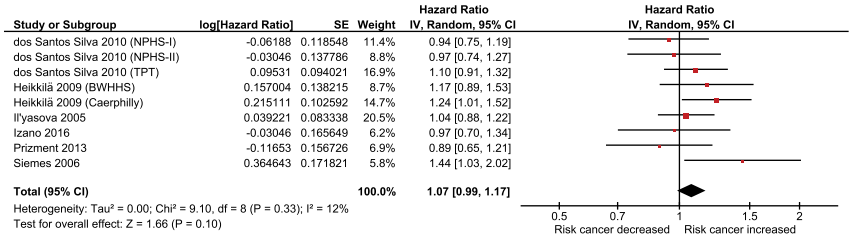


Figure 4. Forrest plot for the association between CRP and colorectal cancer.

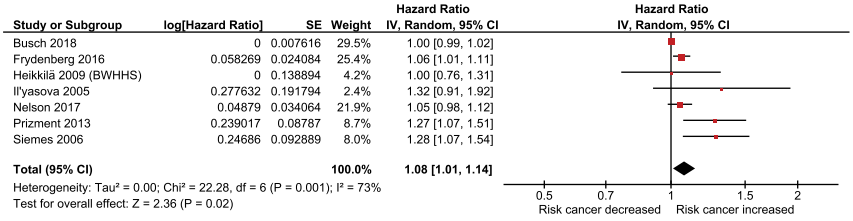


Figure 5. Forrest plot for the association between CRP and breast cancer.

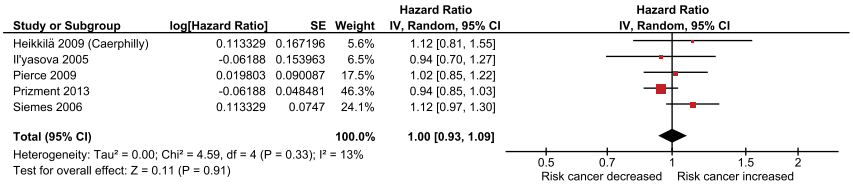


Figure 6. Forrest plot for the association between CRP and prostate cancer.

Discussion

This is the first meta-analysis of prospective, population-based cohort studies that investigated the association between CRP levels and the risk of an incident cancer. It showed that cancer free individuals with higher CRP levels have an increased risk of breast and lung cancer of 8% and 29%, respectively. It further demonstrated a significantly increased risk for any type of cancer of 11%. No significant associations were found for incident colorectal or prostate cancer.

It is well-known that patients with cancer have increased levels of CRP compared to individuals without cancer. [6,9] However, these results come from studies in which CRP levels are measured when the cancer is already present. Then, increased CRP levels may well be the result of an inflammatory response that is generated against the cancer. [6,9] Therefore, previous reviews of Heikkila et al. (2007) and Allin et al. (2011) stated that there was still

too little evidence to answer the question whether inflammation as measured by CRP has a causal role in malignancies, and that large prospective studies were needed to provide an answer to this question. [6,9]

In the past years, several others have published reviews and meta-analyses for subtypes of cancer. Similar to our results, CRP levels were associated with an increased risk of breast cancer with a relative risk (RR) of 1.07 (95% CI: 1.02 – 1.12) and lung cancer with a RR of 1.28 (95% CI: 1.17 – 1.41). [84,85] Although a statistically significant relation between CRP levels and risk of colorectal cancer with a RR of 1.12 (95% CI, 1.01–1.25) has been described, we could not confirm this [86] However, it is important to notice that all these studies combined effect estimates of both nested case-control and cohort studies, even though mathematically ORs and HRs should not be pooled.

In 2013 a meta-analysis of prospective studies studying CRP and incident cancer found comparable results for total cancer incidence and incident lung cancer. [87] They found no association for colorectal and prostate cancer, but contrary to our results, also no statistically significant association for risk of CRP and breast cancer. This could be explained by the fact that in this study different exposure measures (e.g. CRP was measured continuously, in quartiles or clinical cut-offs) were combined.

Overall, when we pool similar exposure measures (e.g. only continuously analysed CRP levels) and similar effect estimates (e.g. only HR) we found a significant association between CRP and any incident cancer and specifically lung and breast cancer. Whether this also means that there is a causal relationship between CRP and cancer, remains to be answered. Four of the studies included in this meta-analysis performed a sub-analysis in which they excluded the first years of follow-up. [8,23,46,57] One study described significant results for risk of any cancer after exclusion of three years of follow-up time. [23] However, in other studies significance was lost or the results were attenuated. [8,46] Regarding lung and breast cancer, for which we found significant associations in this meta-analysis, associations remained significant even when the first 5 years of follow-up were excluded from the analysis. [8,57] In our opinion, from these studies no conclusions can be drawn as to whether these results can not merely be explained by reverse causality.

Some of the included studies also assessed genetic determinants, in which the authors investigated the association between genetic polymorphisms influencing CRP levels and the risk of cancer. Genetic risk scores from multiple SNPs (single-nucleotide polymorphisms) were found to be associated with colorectal cancer. [46,88] Furthermore, CRP SNPs have been found to be associated with lung cancer as an independent risk indicator. [8]

Strengths and Limitations

This is the first meta-analysis of prospective, population-based cohort studies that investigated the association between CRP level and cancer incidence. All included studies were of high quality with a sufficient amount of follow-up time. Previously, reviews and

meta-analyses only included prevalence studies, too few prospective studies, or pooled studies that were not comparable. Although previous meta-analyses of case-control studies showed a significant association between CRP and cancer, the included studies in these meta-analyses were of a retrospective design. Therefore, associations might be explained by reverse causality or bias.

This study has some limitations that warrant mentioning. First, although a large number of prospective studies were included, there was a high variety in the reported exposure and outcome measures. As a result, only a small number of the selected studies could be pooled in the meta-analysis. For greater comparability, we would like to urge future studies to report continuously analyzed CRPs instead of cut-off categories.

Additionally, although this meta-analysis shows that there is a significant association between CRP and cancer, it is still unclear what the nature of this association is. No conclusions can yet be drawn on whether this relationship is causal (meaning CRP directly plays a role in the etiology of cancer), is due to reverse causality or that CRP is a proxy measure for inflammation leading to cancer.

In the future, both these limitations could be solved by performing a patient level meta-analysis.

In conclusion, this meta-analysis of prospective, population-based cohort studies suggests that there is a significant association between CRP level and cancer incidence, specifically lung and breast cancer. A future patient-level-meta-analysis of large prospective studies examining the association of CRP with cancer incidence, would be valuable to determine the role of CRP in the etiology of cancer.

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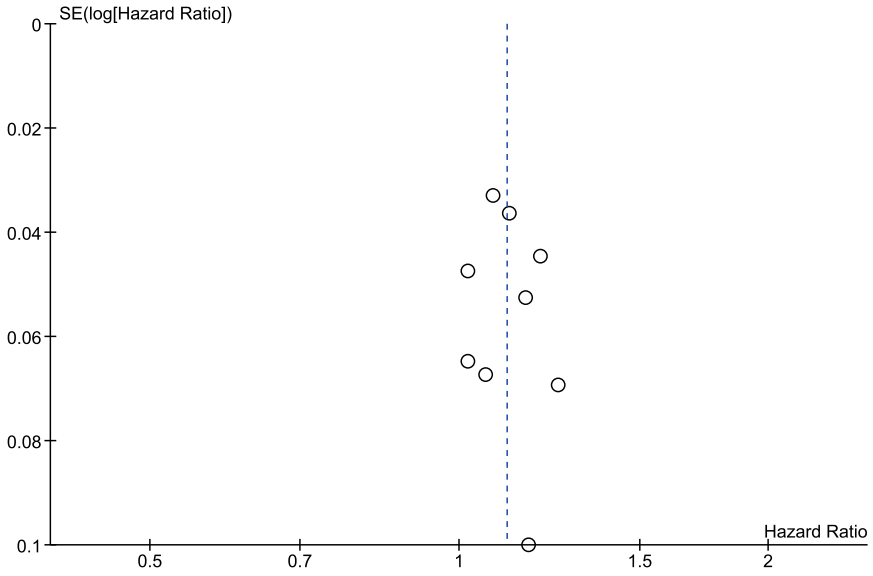
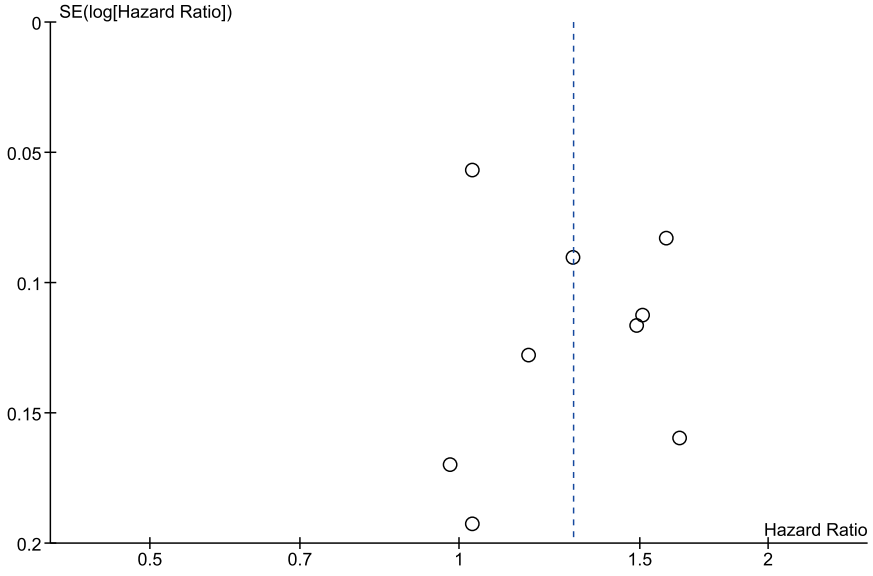
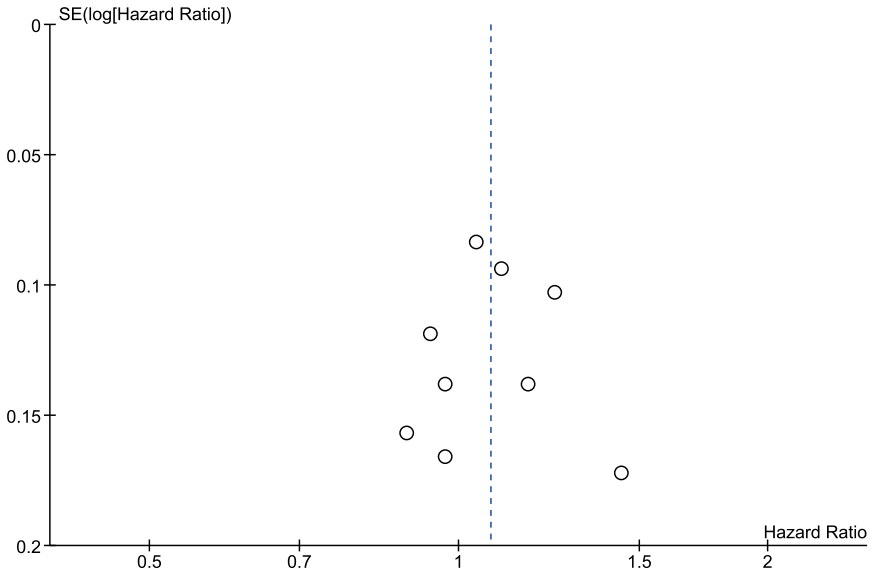


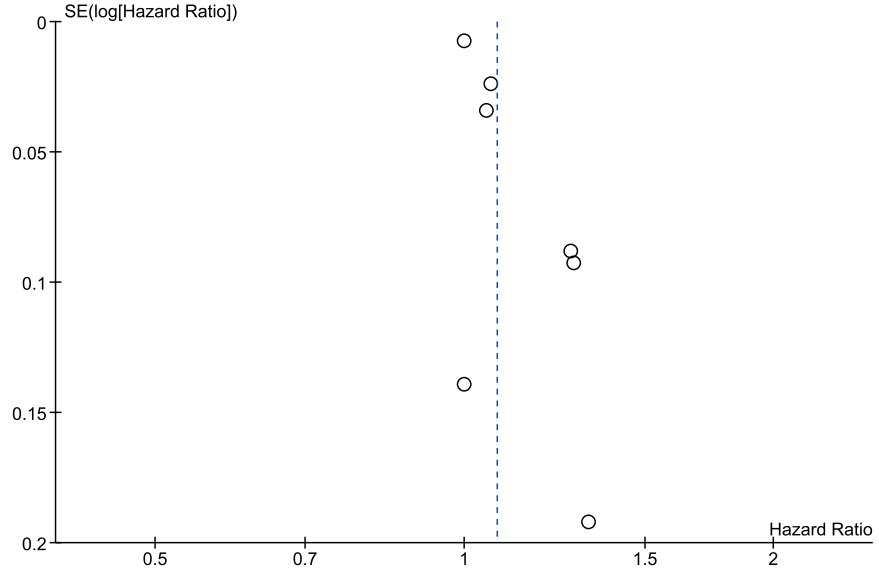
Supplementary Figure 1. Funnel plot for the association between CRP & any solid cancer.



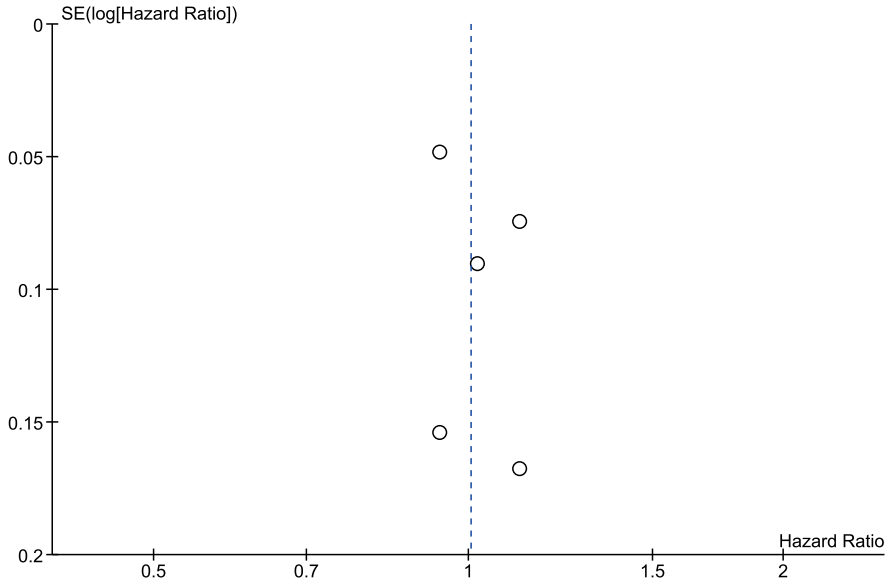
Supplementary Figure 2. Funnel plot for the association between CRP & Lung cancer



Supplementary Figure 3. Funnel plot for the association between CRP & Colorectal cancer



Supplementary Figure 4. Funnel plot for the association between CRP & Breast cancer



Supplementary Figure 5. Funnel plot for the association between CRP & Prostate cancer

Supplementary Materials

embase.com	1851	1818
Medline Ovid	1315	371
Web of science	1376	596
Cochrane CENTRAL	202	75
Google scholar	200	175
Total	**4944**	**3035**

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Chapter 3

Reference values for white blood-cell-based inflammatory markers in the Rotterdam Study: a population-based prospective cohort study.

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Scientific Reports, 2018

Abstract

Background: Novel prognostic inflammatory markers of cancer survival and cardiovascular disease are; the neutrophil-to-lymphocyte ratio (NLR), the platelet-to-lymphocyte ratio (PLR) and the systemic immune-inflammation index (SII). As normal values for these markers are unknown, our objective was to obtain reference values in the general population.

Methods: We obtained data from a population-based prospective cohort study of individuals aged 45 years and over between 2002 and 2014. Absolute blood counts were used to calculate the NLR, PLR and SII. All inflammatory indices followed a lognormal distribution. We calculated the mean and 95% reference intervals in an unselected population. Furthermore we studied whether the inflammatory markers differed between age categories and gender.

Results: In total 8,711 participants (57.1% female; mean age 65.9 years, standard deviation 10.5 years) were included. Mean values and corresponding 95% reference intervals for the NLR were: 1.76 (0.83–3.92), for PLR: 120 (61–239) and for SII: 459 (189–1168). The inflammatory markers increased with age. The PLR and SII were higher in females, whilst the NLR was higher in males.

Conclusion: We provided reference values for new inflammatory markers. All increase with age and vary with gender. This provides context that allows for proper interpretation of their potential value in future clinical practice and research.

Introduction

Low-grade inflammation is associated with important chronic diseases in the elderly such as diabetes, cardiovascular disease and cancer. [1-7] For instance, several immune mechanisms play a role in the formation and activation of atherosclerotic plaques that lead up to cardiovascular disease and the over-expression of TNF-α is associated with insulin resistance and subsequently type 2 diabetes. [2,7] Furthermore chronic inflammation is also since long considered as one of the basic pathogenic processes in cancer development. [3,4] Additionally, it is thought that, once the cancer has developed, the immune system plays an important role in surveillance and elimination of cancer cells. [4]

This has led to the examination of various inflammatory markers and indices as a potential biomarker or prognostic factors.[8] Traditional measures, such as C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) have been extensively studied, previously. [5,6,8] Recently, several new white blood-cell-based inflammatory indices have been introduced as prognostic markers: the neutrophil-to-lymphocyte ratio (NLR), the platelet-to-lymphocyte ratio (PLR) and the systemic immune inflammation index (SII). [9-13]

Both the NLR and PLR are ratios; of the peripheral neutrophil and lymphocyte counts and the peripheral platelet and lymphocyte counts, respectively. The SII has integrated peripheral lymphocyte, neutrophil and platelet counts into one indicator, with the aim to better reflect the balance between the host's inflammatory and immune status.[10] The NLR, PLR and SII can be easily calculated from low-cost and frequently used available measures and are thought to be more specific than CRP or the ESR.

It is generally assumed that the levels of these inflammatory markers are elevated in individuals with cardiovascular disease or cancer. However, normal ranges for the NLR, PLR or SII are unknown and most researchers have estimated cut-off points within their sample population, , resulting in a wide and inconsistent range of cut-off points used in current literature.[12-14]. Reference values are therefore needed to put the results of previous studies into a context that allows for proper interpretation of their potential clinical value. The objective of this study was therefore, to obtain these reference values from the general population in a large and longstanding population-based prospective cohort study.

Methods

Study setting

The analyses were performed in the Rotterdam Study, a long term population based prospective cohort study in the Rotterdam area, the Netherlands. Its rationale and design have been described extensively, previously. [15,16] Briefly, inhabitants of the suburb Ommoord, aged 55 years and older, were invited to participate in 1989. Of the 10,275 invited subjects,

7,983 entered the study (78%). A second cohort of 3,011 persons (67% response), was enrolled between 2000 and 2001. In 2006 a third cohort, with 3,932 persons of 45 years and older, was enrolled (65% response). This resulted in an overall study population of 14,926 individuals, aged 45 years and older.

Participants were visited at home at baseline for a standardized interview on health status. Subsequently, a physical examination followed during a visit at the study centre. These interviews and visits were repeated approximately every four years (**Supplementary Figure 1** [15]). The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports. Informed consent was obtained from all participants. All methods were performed in accordance with the relevant guidelines and regulations.

Definition of study population

White blood cell count, including leucocyte differentials, were only part of the protocol from the fourth visit of the first cohort onwards (**Supplementary Figure 1**). Therefore, for this study we used information from the fourth centre visit of the first cohort (RS-I-4 (January 2002 – July 2004); n = 3,550), the second visit of the second cohort (RS-II-2 (July 2004 – December 2005); n = 2,468) and the baseline visit of the third cohort (RS-III-1 (February 2006 – December 2008); n = 3,932) and onwards. Of the 9,950 eligible participants; 8,912 (89.6%) donated blood. Participants for whom the NLR, PLR or SII could not be calculated, due to missing values (n = 201), were excluded. This resulted in a study cohort of 8,711 individuals (**Figure 1**).

Collection of the samples

Fasting blood samples were collected at the study centre and were stored at -80°C until full blood count measurements. These measurements included absolute counts of granulocytes, lymphocytes and platelets and were performed using the COULTER® Ac·T diff2™ Hematology Analyzer (Beckman Coulter, San Diego, California, USA). In an additional analysis, the normal distribution of hemoglobin and CRP levels were assessed as well. CRP levels were measured using a particle enhanced immunoturbidimetric assay (Roche Diagnostics, Mannheim, Germany).

The neutrophil-to-lymphocyte ratio was calculated on the basis of absolute peripheral granulocyte (as a proxy for the neutrophil count) (N; $x10^9$/Liter) and lymphocyte (L; $x10^9$/Liter) blood counts, using the formula: NLR = N/L. [9]

The platelet-to-lymphocyte ratio was calculated on the basis of peripheral platelet(P; $x10^9$/Liter) and lymphocyte (L; $x10^9$/Liter) blood counts, using the formula: PLR = P/L. [12]

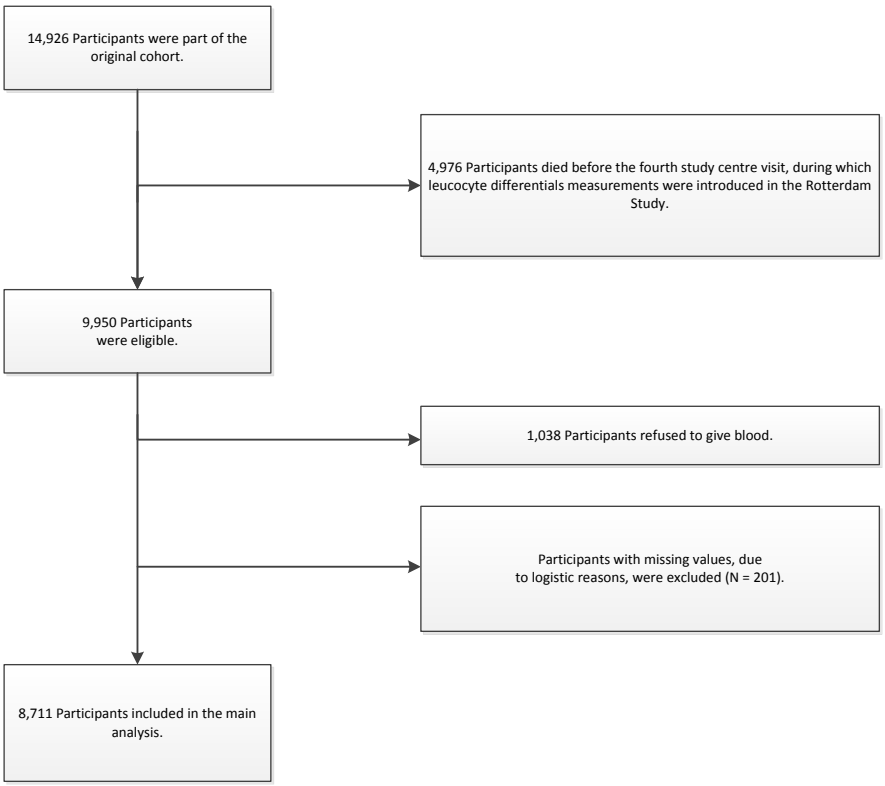

Figure 1. Flowchart of the study population

The systemic immune-inflammation index (SII) was calculated on the basis of peripheral platelet (P; $x10^9$/Liter), granulocyte (N; $x10^9$/Liter) and lymphocyte (L; $x10^9$/Liter) blood counts, using the following formula: SII = P * N/L. [10] All the inflammatory markers are either ratios or indices and as such do not have a unit.

Assessment of other variables

The following individual characteristics were determined at study entry interview or during the visits at the study centre: age, sex, study entry body mass index (BMI; kg/m^2), smoking status (never/former/current), and socio-economic status, based on education level (SES; high [university/higher vocational education] / intermediate [general secondary education/ intermediate vocational education]/ low [lower secondary education/primary education with a higher, but not completed education/primary education]). Status on type 2 diabetes was ascertained either at study entry or during follow-up by use of general practitioners' records (including laboratory glucose measurements), hospital discharge letters, and serum

glucose measurements from the centre visits. [17] Diabetes was defined, in concordance with the WHO guidelines, as a fasting glucose ≥ 7.0 mmol/Liter or use of glucose – lowering medication. [18]

Statistical Analyses

The distribution of the data was visualized by means of histograms and Q-Q plots. Since none of the inflammatory markers were normally distributed and all were slightly skewed to the right (**Figure 2**), we log-transformed them prior to performing any of the analyses. These values were then back-transformed to provide reference values for clinical practice. [19] To present reference values of the inflammatory markers we calculated the 2.5% and 97.5% reference limits in our study population. The 2.5% and 97.5% reference limits reflect the 2.5th and 97.5th percentiles, respectively. Subsequently, the differences between the distribution of the inflammatory markers in females versus males and different age classes [45-54; 55-64; 65-74; 75-84; ≥ 85 years], were assessed using the Student's t-test or ANOVA.

To evaluate whether inflammatory markers indeed truly change with age we used a second measurement in the same individual, which was on average 6.1 years later (range 3.0 – 10.9 years), from the blood draw at RS-I-5 (March 2009 – January 2011); n = 2,147; RS-II-3 (February 2011 – February 2012); n = 1,893 and RS-IIII-2 (March 2012 – June 2014); n = 3,122, respectively (see **Supplementary Figure 1**). Out of the 7,162 living participants, in total 5,849 participants had two measurements available. Differences were assessed using a Paired Samples t-test.

To see whether the distribution was influenced by any current infection, we further assessed the associations in individuals for whom a CRP (mg/Liter) measurement was available (RSIII-1: 3,462). We considered all individuals with a clinically elevated CRP level (CRP > 10 mg/Liter) as having a potential infection and excluded them from the analysis.

All analyses were performed using SPSS software (Version 21.0). Statistical significance of associations was accepted at a *P*-value < 0.05.

Data availability

Data can be obtained upon request. Requests should be directed towards the management team of the Rotterdam Study (secretariat.epi@erasmusmc.nl), which has a protocol for approving data requests. Because of restrictions based on privacy regulations and informed consent of the participants, data cannot be made freely available in a public repository.

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands , implementing the "Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study)". All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

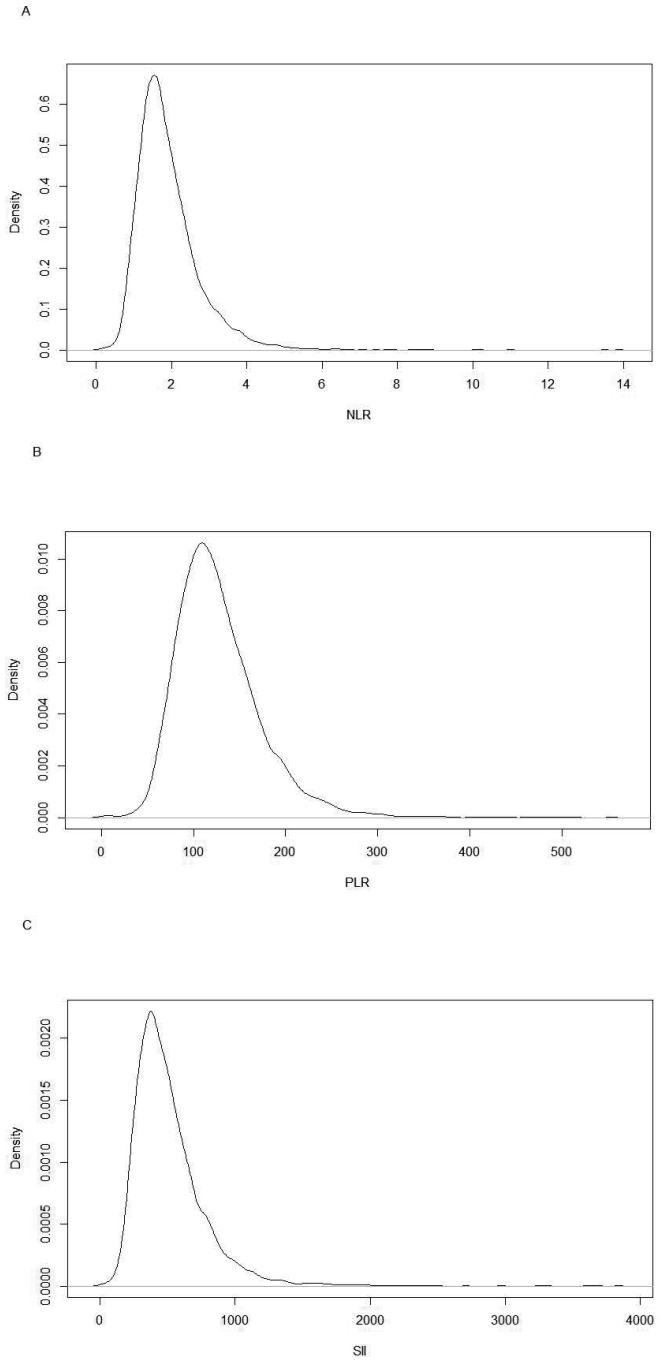

Figure 2. Distributions of the inflammatory markers in the general population. Panel A. NLR Panel B. PLR Panel C. SII.

Results

Main analysis

In total 8,711 participants were included in the analyses for the three inflammatory measures (see **Supplementary Figure 1**). The cohort characteristics are presented in **Table 1.**

The mean NLR in the general population was, 1.76, with a 2.5% limit at 0.83 and 97.5% limit at 3.92. The mean NLR was statistically significantly higher in males (mean of 1.88) than in females (mean of 1.68), *P*-value < 0.001 (see **Supplementary Figure 2**). The mean NLR was generally higher in the higher age categories, with the highest age category > 85 years of age having a mean NLR of 2.13 versus those in the youngest age category of 45-54 years of age of 1.63 (*P*-value < 0.001, **Table 2**). The shape of distribution of the NLR also changed with age, being almost normal for the younger age categories whilst becoming more asymmetrical with age (see **Supplementary Figure 3**). The Skewness statistic and standard error (SE) are: 1.4 (SE: 0.06), 2.2 (SE: 0.05), 2.6 (SE: 0.05), 2.0 (SE: 0.06) and 3.2 (SE: 0.14) for the age categories: 45 – 54 years, 55 – 64 years, 65 – 74 years, 75 – 84 years and ≥ 85 years, respectively.

Similar to the NLR, both the PLR and SII were higher in the higher age categories (*P*-value <0.001 for both). However the PLR and SII were higher in women than in men (*P*-value <0.001 and 0.027, respectively) (see **Table 2, Supplementary Figure 2 and 3**). These results were consistent within the three sub-cohorts separately (data not shown).

To evaluate whether inflammatory markers indeed increase over time, we assessed the change of the inflammatory markers in 5,842 participants with two measurements. At the second blood draw the mean NLR was 1.90 and the mean SII was 465, both significantly higher (Paired Samples t-test: *P*-value <0.001 for both). The mean PLR at the second blood draw was 119 and significantly lower compared to the first blood draw. The median within-person change was for the NLR: 0.10 (IQR: -0.21 – 0.44), for the PLR: -3 (-20 – 14) and for the SII: 19 (-72 – 126).

Sensitivity analyses

To see whether the distribution was influenced by any current infection, we investigated the effect of excluding individuals with an elevated CRP level. CRP measurements were only performed for 3,462 individuals in RS-III-1, of whom in 133 individuals (3.8%) the CRP level was > 10 mg/L and 3,322 (96.0%) individuals had a normal CRP level. Individuals with an elevated CRP level had a significantly higher mean NLR (2.24), PLR (129) and SII (691) compared to those with a normal CRP level; mean NLR (1.61), PLR (117) and SII (444) (Student's t-test: *P*-value for all <0.001). However, removing individuals with an elevated CRP from the population did not affect the mean of the overall population for any of the inflammatory indices. It also only slightly affected the 97.5% limit. When individuals

with a clinically elevated CRP were excluded from the population; the 97.5% limit changed from 3.60 to 3.50 (for the NLR), from 225 to 221 (for the PLR) and from 1112 to 1061 (for the SII), respectively. Individuals with an elevated CRP at the first measurement showed a decrease in the median NLR levels (median -15.5%), whereas for individuals with a normal CRP, the NLR increased with 6.3%.

Table 1. Cohort characteristics.

Characteristic		**Study Cohort**	
		N	%
Total		8,711	100
Sex	Male	3,733	42.9
	Female	4,978	57.1
Age (years)	Mean (SD)	65.9	10.5
Age category (years)	45 – 54	1,474	16.9
	55 – 64	2,780	31.9
	65 – 74	2,573	29.5
	75 – 84	1,583	18.2
	≥ 85	302	3.5
SES	High	1,651	19.2
	Intermediate	3,597	41.9
	Low	3,346	38.9
BMI (kg/m^2)	Mean (SD)	27.1	4.1
Smoking	Current	1,734	20.2
	Former	4,288	49.9
	Never	2,570	29.9
Diabetes Status		952	10.9

SD; standard deviation, SES; socio-economic status, BMI; Body Mass Index
Unknown: SES (117), smoking (119) and BMI (167).
Sex, SES status and BMI at baseline. Age, smoking status and DM status at time of blood draw.

To assess differences between distribution of the inflammatory markers amongst the various covariates we used the Students' t-test or ANOVA (Analysis of Variance). All tests were statistically significant.

Table 2. Reference values for the inflammatory markers.

			NLR	PLR	SII
General Population		mean	1.76	120	459
		2.5% limit	0.83	61	189
		97.5% limit	3.92	239	1168
Sex	Male	mean	1.88	112	453
		2.5% limit	0.88	57	185
		97.5% limit	4.14	230	1168
	Female	mean	1.68	126	463
		2.5% limit	0.80	65	194
		97.5% limit	3.80	246	1169
Age category (years)	45-54	mean	1.63	118	456
		2.5% limit	0.80	62	189
		97.5% limit	3.44	211	1063
	55-64	mean	1.61	116	436
		2.5% limit	0.79	60	186
		97.5% limit	3.53	226	1109
	65-74	mean	1.82	119	455
		2.5% limit	0.86	60	186
		97.5% limit	3.92	239	1131
	75-84	mean	2.02	127	500
		2.5% limit	0.96	61	196
		97.5% limit	4.53	268	1373
	≥ 85	mean	2.13	131	522
		2.5% limit	0.89	63	205
		97.5% limit	5.86	282	1798

NLR = neutrophil-to-lymphocyte ratio = absolute peripheral granulocyte count ($x10^9$/L) / absolute lymphocyte count ($x10^9$/L)

PLR = platelet-to-lymphocyte ratio = absolute peripheral platelets count ($x10^9$/L) / absolute peripheral lymphocyte count ($x10^9$/L)

SII = systemic immune-inflammation index = absolute peripheral granulocyte count ($x10^9$/L) / absolute lymphocyte count ($x10^9$/L) * absolute peripheral platelets count ($x10^9$/L)

Discussion

In the past few years, novel inflammatory markers for prognosis in patients with cancer and cardiovascular disease have been described in the literature. The NLR, PLR and the SII are all composites of blood cell counts, which are standard, low-cost measurements that are already incorporated into daily clinical practice and can be calculated easily from these widely available current measures.

However, the reference limits of these white blood –cell based inflammatory markers in the general population are unknown. Therefore the cut-off values, used for risk assessment, were generally estimated in a clinical sample population consisting of patients with solid tumors. This has resulted in a wide and inconsistent range of cut-off points presented throughout the present literature. To properly evaluate the clinical significance of these new inflammatory markers we need to be able to interpret them in the context of the normal ranges. Knowledge of their distribution and reference values within the general population is therefore essential. This paper provides those reference values, obtained from a large population-based cohort aged 45 years and older.

All inflammatory markers had a skewed (right) distribution. Even when outliers with a clinically elevated CRP were excluded from the population, the distribution in the general population remained asymmetrical. The distributions also did not change when stratified for sex.

However, the distribution of the SII, NLR and PLR was different between age categories (see **Supplementary Figure 3**). This is especially apparent for the distribution of the NLR. The skewed distribution of inflammatory markers in the overall population can largely be attributed to the distribution amongst the higher age categories, whereas the distribution of the NLR amongst the lower age categories is almost normal. We showed that all inflammatory markers increased with age. This resembles the distribution of CRP and the ESR over different age categories. [20,21] Possibly the distribution skews with age, however it is also possible, and perhaps more likely, that its non-symmetry can be attributed to diseases that become more prevalent with age, such as diabetes, cardiovascular disease and cancer. Future research should elucidate the relationship between these inflammatory markers and morbidity in the general population.

Strengths of this study are its prospective nature, its size, and the fact that it is population based. Therefore, we obtained a good estimate of the true normal range of the inflammatory markers within the general population aged 45 years and older and additionally provided insight into the variation of these inflammatory markers. We showed that they increase with age (consistent for all three sub-cohorts) and that the reference values are different for men and women, which is consistent with current literature on CRP and ESR. [20,21] Furthermore, for the NLR and SII we showed that they increase over time.

However, there are some limitations of this study that deserve mentioning. To be able to calculate the inflammatory markers, we needed a differential white blood count. For the absolute neutrophil count we had to take the total granulocyte count as a proxy. However, any misclassification of granulocytes would probably be non-differential and therefore would not have introduced any bias into the results. Another potential limitation is that this measurement was only part of the protocol from the fourth study centre visit of the first cohort onwards, meaning that we have no information on the one-third of the population that had died before that time point. Some participants refused to give blood, meaning that in total about 40% of the original population had to be excluded from this analysis. However, we do not believe that the exclusion of this part of the study population has introduced any bias into this study, as this reflects what happens in the general population. Although the CRP measurements are available for only a part of the population, a sufficient number remains to draw conclusions on the effect of an elevated CRP level on the inflammatory markers.

Lastly the population we examined consisted predominantly of Caucasians (98%) and raises the question whether these results are generalizable towards other ethnic groups. It is known that there are hematologic differences between, for instance, Caucasians and African-Americans.[22-24] Although our results could be used as a bench-mark, we would suggest similar studies amongst different ethnicities to further confirm these new reference values.

In conclusion, this paper provides reference values for three novel prognostic systemic inflammatory markers; the neutrophil-to-lymphocyte ratio, the platelet-to-lymphocyte ratio and the systemic immune-inflammation. This is essential to further evaluate the potential value for clinical practice of these new inflammatory markers.

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Supplementary Figure 1. Diagram of examination cycles of the Rotterdam Study.

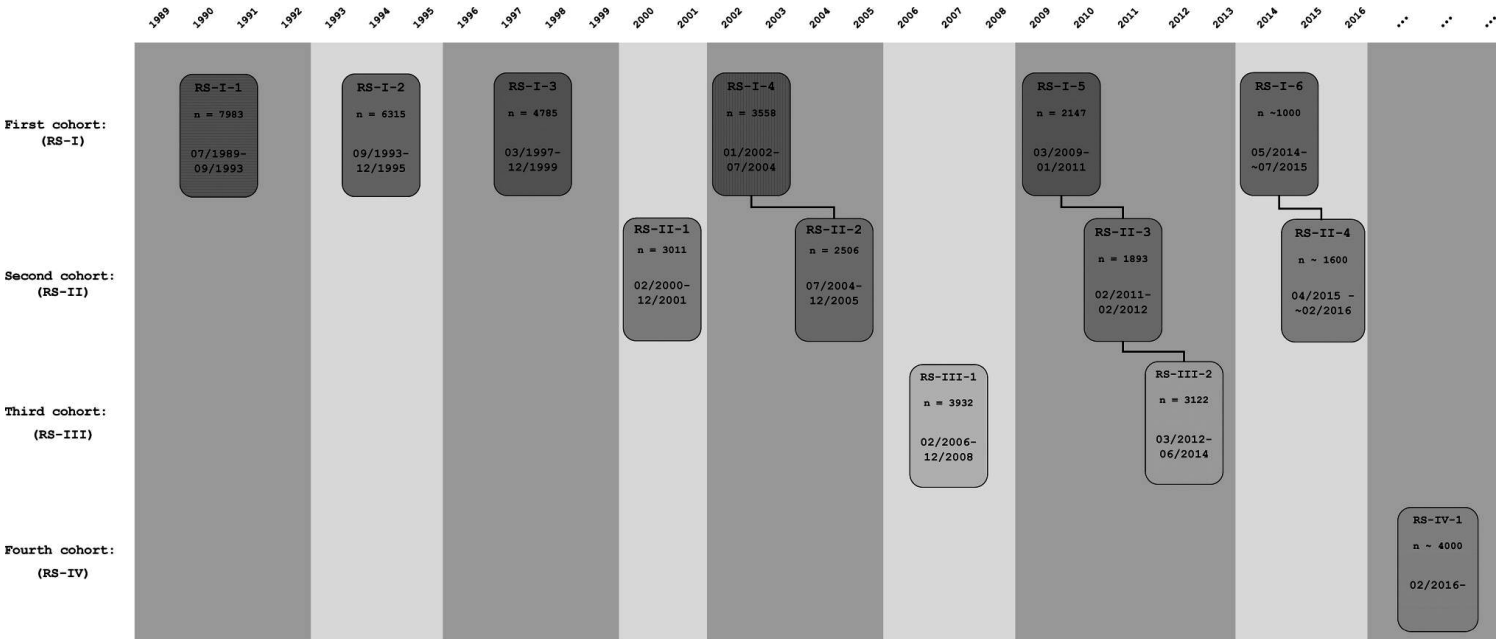
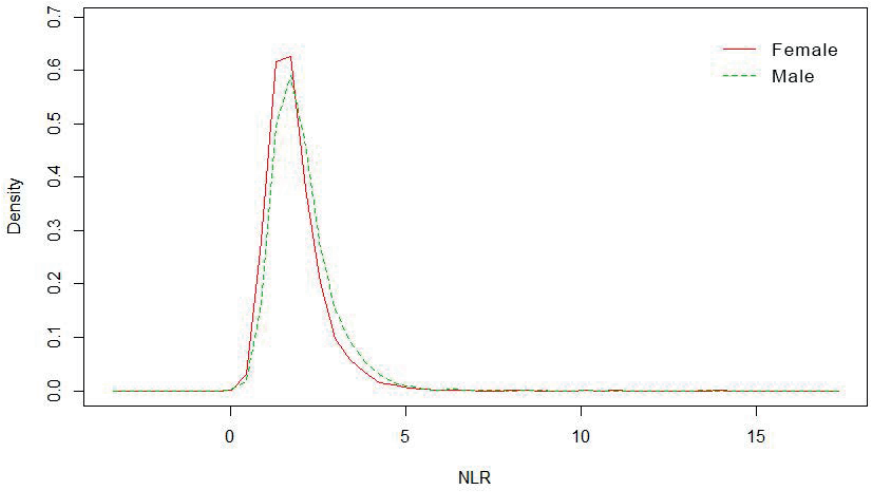


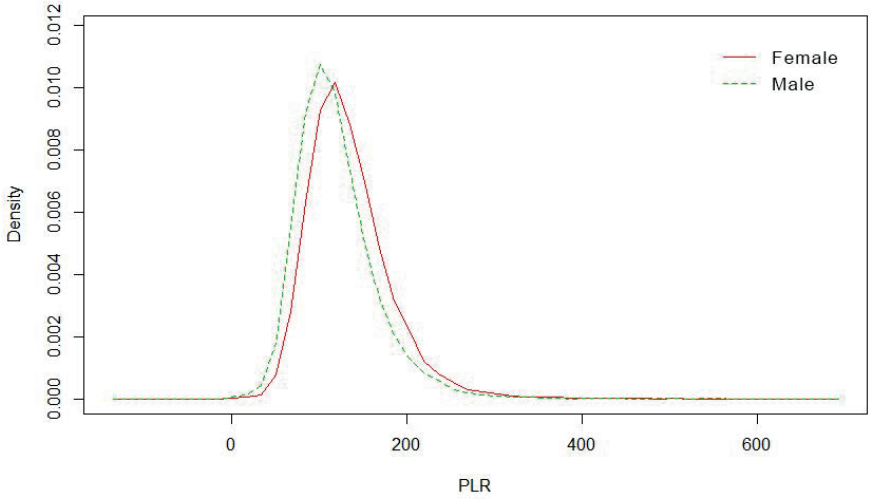
Diagram of examination cycles of the Rotterdam Study (RS). RS-I-1 refers to the baseline examination of the original cohort (pilot phase 07/1989–12/1989; cohort recruitment 01/1990–09/1993). RS-I-2, RS-I-3, RS-I-4, RS-I-5, and RS-I-6 refer to re-examinations of the original cohort members. RS-II-1 refers to the extension of the cohort with persons in the study district that became 55 years since the start of the study or those of 55 years or over that migrated into the study district. RS-II-2, RS-II-3, and RS-II-4 refer to re-examinations of the extension cohort. RS-III-1 refers to the baseline examination of all persons aged 45 years and over living in the study district that had not been examined already (i.e., mainly comprising those aged 45–60 years). RS-III-2 refers to the first re-examination of this third cohort. Examination RS-I-4 and RS-II-2 were conducted as one project and feature an identical research program. Similarly, examinations RS-I-5, RS-II-3, and RS-III-2 share the same program items. Also, examinations RS-I-6 and RS-II-4 are conducted as one project. RS-IV-1 refers to the baseline visit of a new cohort, to be established in February 2016.

Supplementary Figure 2. Distributions of the inflammatory markers stratified for gender.

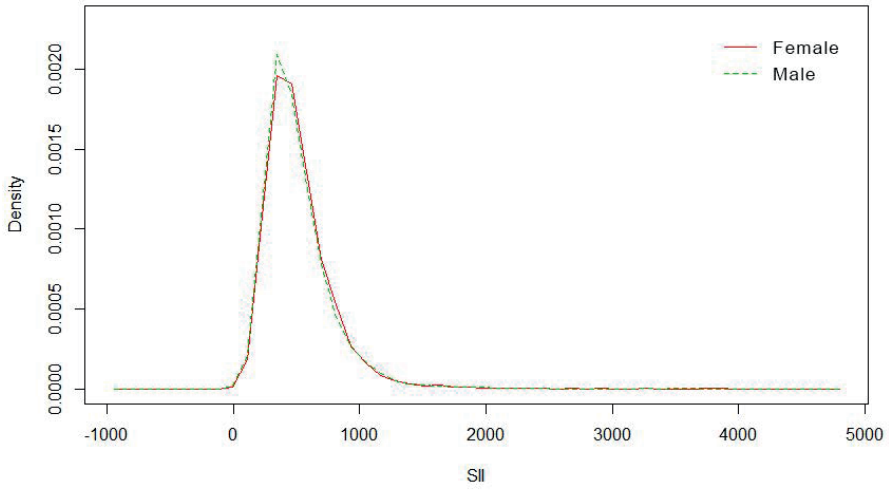
A. NLR



B. PLR

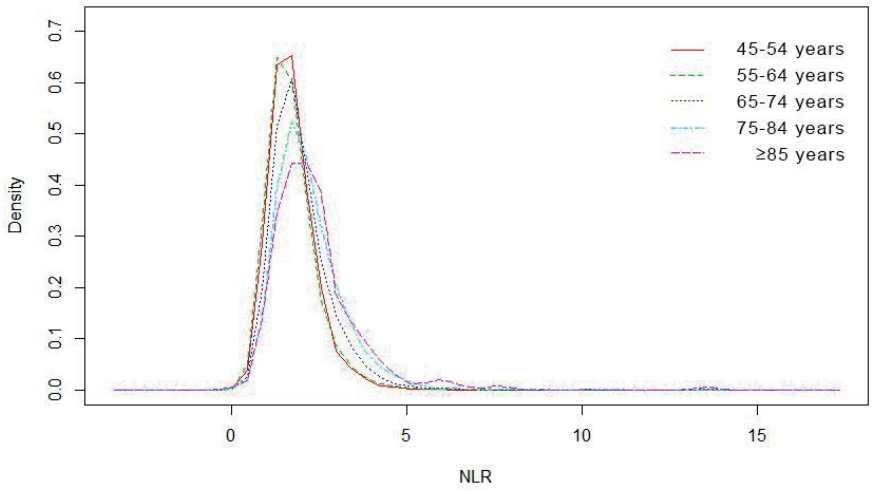


C. SII

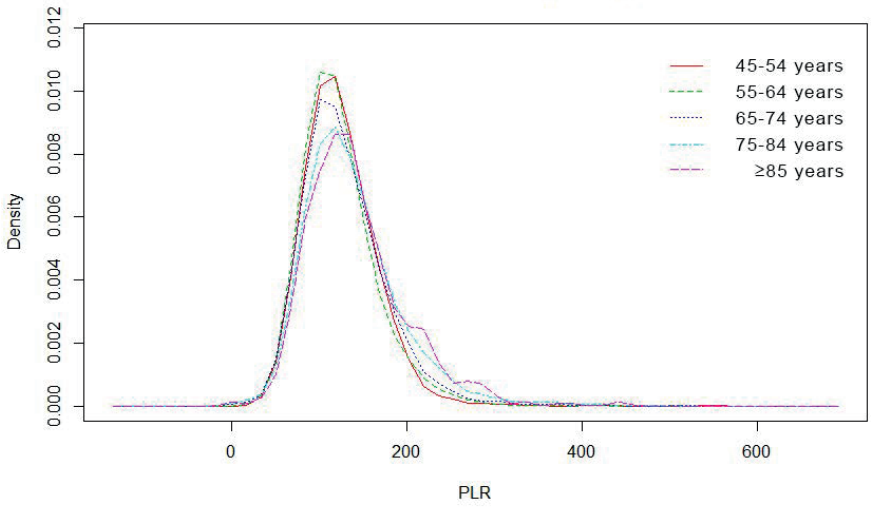


Supplementary Figure 3. Distributions of the inflammatory markers across age categories.

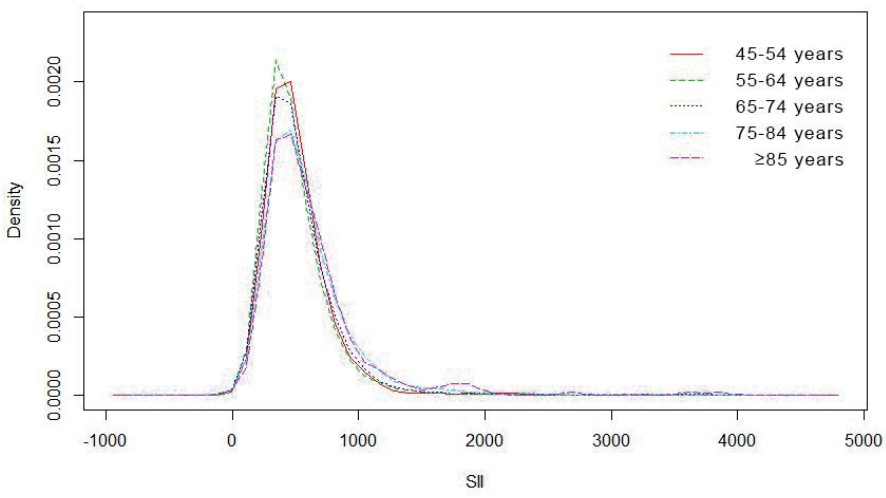
A. **NLR**



B. **PLR**


Density
0.000
0.002
0.004
0.006
0.008
0.010
0.012
0
200
400
600
PLR
45-54 years
55-64 years
65-74 years
75-84 years
≥85 years

C. **SII**


Density
0.0000
0.0005
0.0010
0.0015
0.0020
-1000
0
1000
2000
3000
4000
5000
SII
45-54 years
55-64 years
65-74 years
75-84 years
≥85 years

Chapter 4

The neutrophil-to-lymphocyte ratio as an independent predictor of overall and cause-specific mortality: results from the Rotterdam Study

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Abstract

Background: Inflammation is a risk factor for morbidity and mortality in the elderly. The neutrophil-to-lymphocyte ratio (NLR) is a marker of systemic inflammation that integrates the information of the leukocyte differentials into one variable. We aimed to assess whether the NLR is a risk indicator for overall and cause-specific mortality in the general population.

Methods: We analyzed data (2002-2014) from the Rotterdam Study, a long-standing, population-based, prospective cohort study in a community-dwelling ageing population. The association between the NLR and time to all-cause mortality was assessed with Cox proportional hazard models. We additionally assessed cardiovascular, cancer and other mortality. The multivariable analyses were adjusted for age, gender, socio-economic status (SES), smoking status, body mass index, type 2 diabetes, and history of cancer and cardiovascular disease (CVD).

Results: Data of 8,715 individuals were included. The mean age was 65.9 years (SD 10.5) and the majority were women (57.1%). The NLR was higher in men, higher age categories, smokers and among individuals with lower SES, prevalent diabetes, or a history of cancer or CVD. During the 11.7 years follow-up period, 1,641 individuals died. Survival among individuals in the 3rd, 4th, and 5th quintile of the NLR was significantly poorer than that of those in the 1st quintile (P <0.001). In the multivariable analysis, NLR levels were independently and significantly associated with an increased risk of all-cause mortality (HR: 1.64; 95%CI: 1.44 – 1.86), cardiovascular mortality (HR 1.92; 95% CI: 1.49 – 2.48), and other mortality (HR: 1.86; 95% CI: 1.54 – 2.24). No significant association was found for cancer mortality (HR: 1.20; 95% CI: 0.95 – 1.51).

Conclusion: The NLR is a strong and independent risk indicator for mortality in the elderly population. Its clinical value needs to be established in further studies.

Introduction

Inflammation is considered an important risk factor for morbidity and mortality in the elderly. It is still largely unclear whether we may speak of a causal relation between inflammation and mortality, or whether the inflammation is a manifestation of an underlying illness that causes early death. Moreover, the inflammatory markers are known to increase with age, therefore an elevation of these markers may also be 'part of the process of ageing'. [1]

C-reactive protein (CRP) has been extensively studied as a marker of inflammation and more specifically as a risk indicator for cardiovascular, cancer, and all-cause mortality. [2-5] Nevertheless, no conclusive evidence has been found on its potential causal role in mortality of any cause and its clinical use for early identification of patients at risk of cardiovascular disease. [2,4,6] Furthermore, CRP is likely to be just one of many different elements in the inflammatory pathway.

In an attempt to gain more insight into the relationship between inflammation and mortality, also the total leukocyte count has been studied. It has previously been shown that it is related to cardiovascular, cancer, as well as all-cause mortality. [7-10] However, the total leukocyte count encompasses several cell types, such as granulocytes, lymphocytes and monocytes, which potentially all play a different role. [11] Granulocytes, as a whole, or more specifically neutrophils, are associated with a negative influence on survival, whereas lymphocytes are considered to have protective effects on survival. [11-13] While analyzing them together would not appreciate the opposite roles they seem to have, analyzing them apart would not account for the interaction between these subtypes in their association with mortality.

The neutrophil-to-lymphocyte ratio (NLR) is a composite marker of absolute peripheral neutrophil and lymphocyte counts, which can be used to study the effects of both simultaneously. [14] It is a well-studied marker for survival in patients with cancer and in patients with cardiovascular disease. [13,14] However, it is unknown whether it also is predictive of cancer, cardiovascular, or all-cause mortality in the general population. To this end we studied the NLR and its potential association with overall and cause-specific mortality within the context of the Rotterdam Study; a long-standing, population-based, prospective cohort study among a community-dwelling ageing population, with detailed information on illness and risk factors for chronic disease. We hypothesized that an increased NLR is independently associated with mortality in apparently healthy individuals.

Methods

Study design and population

The rationale and design of the Rotterdam Study have previously been described. [15,16] Briefly, from 1989-1993, inhabitants of the suburb of Ommoord in the city of Rotterdam, aged 55 years and older, were invited to participate. Of 10,275 invited subjects, 7,983 participated (78%). A second cohort of 3,011 persons, also aged 55 years and older, (response: 67%) was enrolled in the years 2000 and 2001. In 2006, the study was again extended with 3,932 persons aged 45 years and older (response: 65%). This resulted in an overall study population of 14,926 individuals aged 45 years and above.

Baseline NLR values were calculated at the earliest study center visit at which a leukocyte differential count was available: the fourth visit of the first cohort (2002 – 2004; n = 3,550), the second visit of the second cohort (2004 – 2005; n = 2,468) and the first visit of the third cohort (2006 – 2008; n = 3,932).

Individuals who had not proved consent for blood draw (N = 1,038) were excluded as well as individuals with missing granulocyte, lymphocyte or platelet counts (N = 197).
The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports.

Assessment of the neutrophil-to-lymphocyte ratio

Fasting blood samples were collected at the study center and full blood count measurements were performed immediately after blood draw. These measurements included absolute counts of granulocytes and lymphocytes and were performed using the COULTER® Ac·T diff2™ Hematology Analyzer (Beckman Coulter, San Diego, California, USA).

The neutrophil-to-lymphocyte ratio (NLR) was calculated on the basis of absolute peripheral granulocyte (as a proxy for the absolute neutrophil count) (N; $x10^9$/Liter) and lymphocyte (L; $x10^9$/Liter) blood counts, using the formula: NLR = N/L. [14]

The NLR was non-normally distributed and therefore log-transformed prior to performing any of the analyses.

Assessment of other covariates

Data on the following known independent prognostic factors of mortality were collected at baseline: age, gender, socio-economic status (SES; based on education level [high/intermediate/low]), baseline body mass index (BMI; kg/m^2), smoking status [never/former/current], prevalent type 2 diabetes status (DM; based on a fasting plasma glucose level of ≥ 7.0 mmol/L (≥ 126 mg/dL) or non-fasting plasma glucose level of ≥ 11 mmol/L (≥

200 mg/dL) or use of blood glucose medication), history of cancer (based on pathology), and lastly, history of cardiovascular disease, including transient ischemic attacks (TIA), stroke (CVA), myocardial infarction (MI), and coronary revascularization (percutaneous transluminal coronary angioplasty or coronary artery bypass grafting). [17-19] High-sensitivity CRP measurements (mg/ml; using a particle enhanced immunoturbidimetric assay, Roche Diagnostics, Mannheim, Germany) were available in a subgroup of the study.

Assessment of outcome

The main outcome of this study was time to all-cause mortality. Dates of death were obtained through the mortality registry of the municipality and the causes of death were obtained from general practitioners' records or hospital discharge letters. The causes of death were coded independently by two physicians according to the ICD- 10 and the ICPC-2. [20,21]

Statistical Analysis

For each participant, follow-up started at the day of inclusion and ended at the date of death or end of the study period (1st of January 2014), whichever came first.

Participants were divided into five groups based on the level of the NLR calculated at baseline. Differences between the five groups were assessed with ANOVAs for normally distributed continuous variables and χ^2-tests for categorical variables. Kaplan – Meier plots were calculated for quintiles and extreme quantiles of the NLR and compared with Log-Rank tests.

Proportional hazard models were used to assess the association between the NLR levels at baseline (continuously and in quartiles) and time to all-cause mortality. Subsequently we assessed the association for cardiovascular and cancer mortality, respectively.

For most variables the proportional hazard assumption did not hold. Therefore, follow-up time was divided into five strata (< 2 years, 2-4 years, 4-6 years, 6-8 years and > 8 years). For example: an individual with an event after 5.4 years follow-up, contributed follow-up time to the first (2 years), second (2 years) and third stratum (1.4 years). The risk of mortality in the last stratum is therefore conditional upon the survival up until that time. [22] We also performed a traditional proportional hazard regression, the results of which can be interpreted as the averaged risks over time. [22]

For 5,421 individuals we had a second measurement available, which we included in a multiple measurements analysis using a time-varying covariates in a Cox model. [23]

All potential confounders, mentioned above, were assessed individually and were included in the multivariable model when they changed the point estimate by more than 10% or were considered as clinically relevant. [24] The results are reported as hazard ratios (HR) and 95% confidence intervals (CI). Effect modification was assessed for smoking by

adding an interaction variable to the model and was considered statistically significant at a P-value < 0.10. We tried to quantify the presence of any unknown and therefore unmeasured confounding through calculating the E-Value. [25]

All statistical analyses were performed using SPSS software (Version 21.0) and R (Version 3.1.3); significance was accepted for two-sided P-values at < 0.05.

Results

Population characteristics

Data of 8,715 participants were included in the analyses (see **Supplementary Figure 1**). The mean age was 65.9 years; the majority were women (4,980; 57.1 %, see **Table 1**). During an average follow-up period of 7.7 years (maximum follow-up period was 11.7 years), a total of 1,641 (18.2%) participants died, of whom 496 from the consequences of cancer (30.2%) and 401 from cardiovascular disease (24.4%). The remaining 45.4% died from another cause such as: chronic obstructive pulmonary disease (COPD), a pneumonia, as a consequence of an accidental fall or multi-comorbidity including Parkinson's Disease and Alzheimer's Disease.

Baseline characteristics for the total population and for each quintile of the NLR can be found in **Table 1**. In summary, the male gender, a higher age, a lower SES, smoking habit, prevalent diabetes, prior cancer diagnosis, and a history of cardiovascular disease were all associated with a higher NLR.

Main outcome

The overall survival was poorer for participants in the higher quintiles of the NLR than for those in the lowest one (Logrank test: *P*-value <0.001, see **Figure 1A**). Survival of participants in the 2nd quintile was not significantly different from that of participants in the 1st quintile (reference), but for other quintiles it did differ significantly. In a further analysis which was restricted to the highest quintile, survival for the 1% with the highest NLR levels was worst (Logrank test: *P*-value <0.001, see **Figure 1B**).

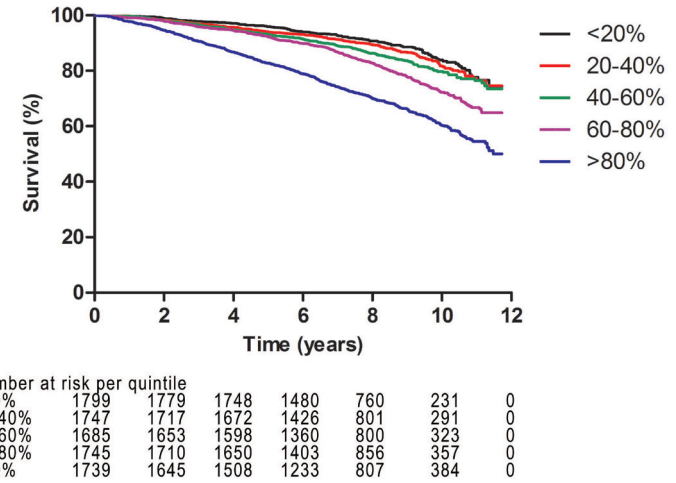
Multivariable analysis showed that the NLR was independently associated with all-cause mortality, after adjusting for age, gender, SES, BMI, smoking, DM, and history of CVD and cancer. The effect of the NLR was not modified by smoking. On average the risk was increased by 64% (HR 1.64; 95% CI: 1.44 – 1.86). The E-values for this analysis were 2.17 for the point estimate and 1.89 for the confidence intervals, respectively. The observed HR of 1.64 could be reduced to 1.00 if there was an unmeasured confounder with a risk of 2.17 or above.

Table 1. Baseline characteristics for each quintile of the NLR.

Characteristic		Neutrophil-to-Lymphocyte Ratio						
		Total	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	**P-value**
			< 1.30	1.30 – 1.59	1.60 – 1.91	1.92 – 2.41	> 2.41	
	Total	8715	1799	1747	1685	1745	1739	
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	
Gender	Male	3735 (42.9)	595 (33.1)	687 (39.3)	703 (41.7)	837 (48.0)	913 (52.5)	<0.001
	Female	4980 (57.1)	1204 (66.9)	1060 (60.7)	982 (58.3)	908 (52.0)	826 (47.5)	
Age (years)	Mean (SD)	65.9 (10.5)	63.2 (9.7)	64.2 (9.5)	65.4 (10.5)	66.8 (10.6)	70.1 (10.6)	<0.001
Smoking *	Current	1734 (19.9)	300 (16.7)	341 (19.5)	365 (21.7)	375 (21.5)	353 (20.3)	<0.001
	Former	4291 (49.2)	854 (47.5)	860 (49.2)	792 (47.0)	876 (50.2)	909 (52.3)	
	Never	2571 (29.5)	627 (34.9)	527 (30.2)	513 (30.4)	458 (26.2)	446 (25.6)	
SES *	High	1652 (19.0)	369 (20.5)	333 (19.1)	321 (19.1)	332 (19.0)	297 (17.1)	0.001
	Intermediate	3598 (41.3)	789 (43.9)	718 (41.1)	690 (40.9)	717 (41.1)	684 (39.3)	
	Low	3348 (38.9)	612 (34.0)	683 (39.1)	654 (38.8)	666 (38.2)	733 (42.2)	
BMI * (kg/m^2)	Mean (SD)	27.1 (4.2)	27.0 (3.9)	27.1 (4.1)	27.3 (4.1)	27.1 (4.2)	26.9 (4.3)	0.081
DM status	Yes	952 (10.9)	145 (8.1)	169 (9.7)	179 (10.6)	221 (12.7)	238 (13.7)	<0.001
	No	7763 (89.1)	1654 (91.9)	1578 (90.3)	1506 (89.4)	1524 (87.3)	1501 (86.3)	
History of cancer	Yes	688 (7.9)	131 (7.3)	131 (7.5)	124 (7.4)	121 (6.9)	181 (10.4)	0.001
	No	8027 (90.9)	1668 (92.7)	1616 (92.5)	1561 (92.6)	1624 (93.1)	1558 (89.6)	
History of CVD	Yes	789 (9.1)	106 (5.9)	121 (6.9)	143 (8.5)	159 (9.1)	260 (15.0)	<0.001
	No	7926 (90.1)	1693 (94.1)	1626 (93.1)	1542 (91.5)	1586 (90.9)	1479 (85.0)	

Unknown: Smoking (119; 1.4%), SES (117; 1.3%) and BMI (167; 1.9%). SES = socio-economic status, DM = Diabetes Mellitus type 2, CVD = cardiovascular disease. Differences between the five groups were assessed with ANOVAs for normally distributed continuous variables and -tests for categorical variables.

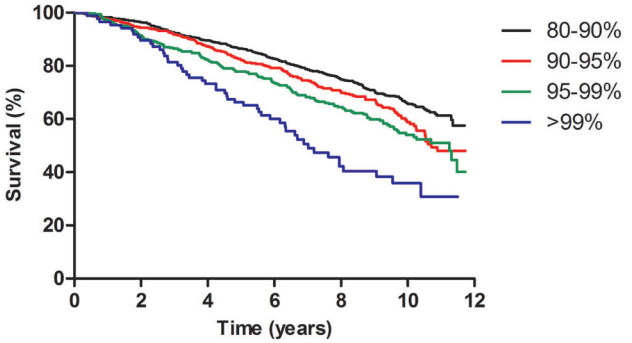
A



Number at risk per quintile

<20%	1799	1779	1748	1480	760	231	0
20-40%	1747	1717	1672	1426	801	291	0
40-60%	1685	1653	1598	1360	800	323	0
60-80%	1745	1710	1650	1403	856	357	0
>80%	1739	1645	1508	1233	807	384	0

B



Number at risk in the highest quintile

80-90%	880	849	788	653	424	211	0
90-95%	425	401	371	307	208	89	0
95-99%	348	318	286	233	151	73	0
>99%	86	77	63	40	24	11	0

Figure 1. A. Kaplan – Meier curves for all-cause mortality for each quintile of the NLR (*P*-value < 0.001. B. Kaplan – Meier curves for all-cause mortality for the highest quintile of the NLR (*P*-value < 0.001)

Table 2. Cox proportional hazard regression for the association of the NLR and all-cause mortality.

Events/cohort	NLR	HR	Lower 95% CI	Upper 95% CI
1,551/8,352	Logtransformed	1.64	1.44	1.86
226/2,107	Q1	reference	-	-
274/2,073	Q2	1.05	0.88	1.25
374/2,082	Q3	1.13	0.96	1.33
677/2,090	Q4	1.59	1.37	1.86

Adjusted for: gender, age in years, SES (socio-economics status: high/intermediate/low), smoking status (current/former/never), BMI (body mass index: kg/m^2), DM (type 2 diabetes mellitus status), history of cancer and history of cardiovascular disease.

In a sensitivity analysis in which we allowed the NLR the change over time for individuals with a second measurement, the averaged risk for all-cause mortality was increased with 68% (HR: 1.68; 95% CI; 1.48 – 1.90) in the fully adjusted model.

The hazard ratio was highest within the first two years after baseline, in which individuals with a higher NLR level at baseline had a more than twofold risk to die of any cause (HR 2.07, 95% CI: 1.47 – 2.90). The hazard ratio gradually decreased over time, but the NLR remained. associated with an increased risk, albeit non-significantly, of 31% for those with a follow-up time of > 8 years (HR 1.31, 95% CI: 0.99 – 1.73) (see **Figure 2**).

Subsequently, we assessed whether the association between baseline inflammatory markers and mortality was attenuated by CRP. A CRP measurement was available for 3,457 individuals from RS-III. CRP levels were independently associated with all-cause mortality, but the association was no longer significant when the NLR was also added to the multivariable model. The point estimate of the NLR was not attenuated by adding CRP to the model (see **Table 3**).

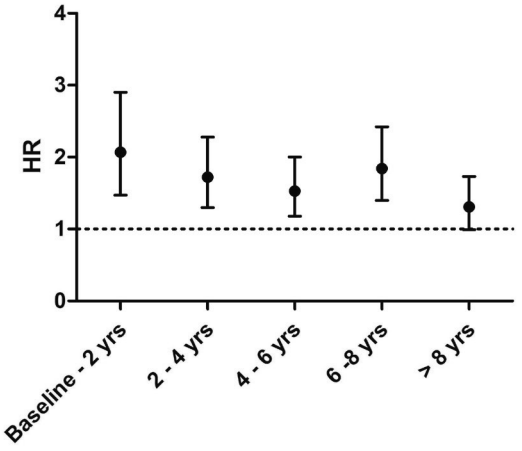


Figure 2. Risk of NLR-related all-cause mortality over time.
Adjusted for: sub-cohort, gender, age (in years), socio-economic status (high/intermediate/low), smoking status (current/former/never), BMI (body mass index, kg/m^2), prevalent type 2 diabetes mellitus, history of cardiovascular disease and history of cancer. Risk for each time stratum were for: baseline – 2 years (HR 2.07, 95% CI: 1.47 – 2.90), 2 – 4 years (HR 1.72, 95% CI: 1.30 – 2.28), 4 – 6 years (HR 1.53, 95% CI: 1.18 – 2.00), 6 – 8 years (HR 1.84, 95% CI: 1.40 – 2.42) and > 8 years (HR 1.31, 95% CI: 0.99 – 1.73).

Table 3. Cox proportional hazard regression for the association of the NLR and all-cause mortality, additionally adjusted for CRP, in a sub-population of the cohort.

Clinical Variable		Main model + NLR			Main model + CRP			Main Model + NLR + CRP		
		HR	Lower 95% CI	Upper 95% CI	HR	Lower 95% CI	Upper 95% CI	HR	Lower 95% CI	Upper 95% CI
Female		0.75	0.52	1.08	0.72	0.50	1.04	0.78	0.54	1.13
Age (in years)		1.09	1.07	1.11	1.10	1.08	1.12	1.09	1.07	1.11
SES	High	reference			reference			reference		
	Intermediate	1.27	0.76	2.12	1.21	0.73	2.03	1.21	0.72	2.02
	Low	1.74	1.03	2.93	1.57	0.93	2.66	1.56	0.92	2.64
Smoking	Never	reference			reference			reference		
	Former	2.04	1.20	3.49	1.96	1.14	3.36	1.94	1.29	3.32
	Current	3.38	1.93	5.93	3.47	1.97	6.11	3.35	1.90	5.91
History cancer		2.56	1.57	4.17	2.60	1.59	4.25	2.59	1.58	4.23
DM		1.37	0.83	2.26	1.44	0.87	2.39	1.44	0.87	2.38
BMI (in kg/m^2)		0.98	0.94	1.02	0.98	0.94	1.02	0.98	0.94	1.02
NLR		2.04	1.31	3.20	-	-	-	1.92	1.19	3.10
CRP (in mg/ml)		-	-	-	1.20	1.02	1.42	1.12	0.94	1.33

For 3,457 individuals from RS-III we had a CRP measurement available, we added CRP to the model to see whether the association between the NLR and the all-cause mortality was attenuated. In RS-III in total 129 individuals died. Proportional hazard assumptions were tested separately in this sub-population and upheld for all variables. History of CVD was neither a significant predictor nor a confounder in this subpopulation and was therefore not included in the model.

HR = Hazard Ratio, SES = socio-economic status, BMI = Body Mass Index, NLR = neutrophil-to-lymphocyte ratio, CRP = C-reactive protein, CVD = cardiovascular disease

Sub-analyses

Additionally, we addressed cause-specific mortality, assessing possible associations between the NLR at baseline and risk of cardiovascular-, cancer- and, other mortality. The risk for cardiovascular mortality was significantly increased and relatively constant over time, with an average HR of 1.92 (95% CI: 1.49 – 2.48) (**Supplementary Figure 2.A.**). In contrast, no significantly increased risk was observed for cancer related mortality, with an average HR of 1.20 (95% CI: 0.95 – 1.51) (**Supplementary Figure 2.B.**). For other mortality the average risk was significantly increased by 86% (HR: 1.86; 95% CI: 1.54 – 2.24). It was highest in the first 2 years with a HR of 4.28 (95% CI; 2.44 – 7.51) and decreased over time to a 31% higher, albeit statistically non-significant, risk for individuals with a follow-up time > 8 years (HR: 1.31, 95% CI: 0.90 – 1.91) (**Supplementary Figure 2.C.**).

Discussion

Previous studies have shown that the NLR is a prognostic marker for mortality in patients with cardiovascular disease and cancer. [13,14] We hypothesized that the NLR was independently associated with mortality in apparently healthy individuals. To our knowledge, this is the first study confirming this hypothesis of an independent relationship between the NLR and early mortality in the general population.

Multiple studies have investigated the association between the WBC count or the leukocyte differentials and all-cause mortality, but none studied individual cell types in relationship to each other. The NLR integrates the information obtained from the leukocyte differentials and provides the opportunity to simultaneously study the association between neutrophils and all-cause mortality and that between lymphocytes and mortality.

Our results are largely in agreement with the results previously found for the association between the WBC count and overall mortality. The WBC count has been consistently associated with both total and cardiovascular mortality. In our study, the association of the NLR with cancer mortality was much weaker and non-significant. Although this might seem counterintuitive because of the prognostic role of the NLR in people with cancer, it is not unexpected as cancer mortality largely depends on available therapeutic options and cancer type. Again this is consistent with literature on the WBC count and cancer mortality in the general population. [26,27]

The effects are controlled for important confounders such as smoking and a higher BMI or comorbidities, such as a history of cardiovascular disease, diabetes or a history of cancer. It is known that the leukocyte and neutrophil counts are also higher in smokers. [7,28] We indeed found that smoking is an important confounder. The association remains robust, however, after adjustment for this factor, which implies that only part of the association between the NLR and mortality is explained by smoking. Moreover, there was no effect

modification of smoking, meaning that the magnitude of the association was not different in smokers compared to non-smokers. Furthermore, the NLR proved the strongest risk indicator when both CRP and the NLR were included in the model. This means that the association was independent from the relationship between CRP and mortality, which suggests that a potential inflammatory pathway that is explained by CRP, is different from the pathway than the one represented by the NLR.

We tried to quantify the presence of any unknown and therefore unmeasured confounding through calculating the E-Value. [25] Although any residual confounding cannot be completely ruled out, we found that any unknown confounder would have to have a risk of 2.17 or above to explain the observed effect. Considering the large number of confounders we have adjusted for, we believe it is unlikely that the effects in this study can be explained by such strong residual confounding.

Overall, our findings seem to confirm that there is an independent relationship between inflammation and mortality. What the nature of this association is, remains uncertain. Although the relationship might be etiological, it may also be that the NLR is a proxy measure of the ageing process or rather a manifestation of an underlying disease.

Consistent with this latter hypothesis, we found that the NLR-related risk of mortality was highest for the first two years of follow-up and decreased over time. This is explained by the effects seen for other mortality (see **Supplementary Figure 2.C.**) and may be a result of a depletion of individuals with an underlying illness or poor health status. However we controlled for history of cancer and cardiovascular disease and even when the first 8 years of follow-up are excluded, the association still persists, making underlying disease a less likely explanation.

Another explanation may be that of a causal association. For instance, it is known that neutrophils infiltrate atherosclerotic plaques and may play a role in the rupture, resulting in a cardiovascular incident.[29] However, this would mean there is an intermediate between the NLR and mortality and that neutrophils play no role in the actual process of dying.

The last explanation would be that the immune system gets damaged as part of the ageing process and that the NLR is a proxy marker for this biological phenomenon.

Strengths and limitations

A large population-based and prospective cohort study such as the Rotterdam Study, with a long follow-up period and detailed information on prevalent disease and important risk factors, is the design of choice for studying associations between blood levels of inflammatory markers and all-cause mortality.

The NLR is derived from the leukocyte differentials, which is a stable, well-standardized and inexpensive measurement that reflects systemic inflammation. Still, cut-off values to stratify patients into currently unidentified risk groups are still lacking. These cut-off values are necessary to evaluate the clinical utility of the NLR.

Another limitation of this study is the fact that the total granulocyte count served as a proxy for the total neutrophil count. We assume, however, that this has had little impact on the results as neutrophils are by far the most abundant type of granulocytes. [30] Any resulting misclassification could have led to an overestimation, but it has been conclusively shown that the associations for granulocytes and neutrophils have the same direction and the same effect size. [12] We believe the obtained effect measures are a fair representation of the true effect.

In conclusion, we have demonstrated that the NLR is independently associated with all-cause mortality in the elderly population, after adjustment for traditional risk factors. Its potential value in clinical practice needs to be established in further studies.

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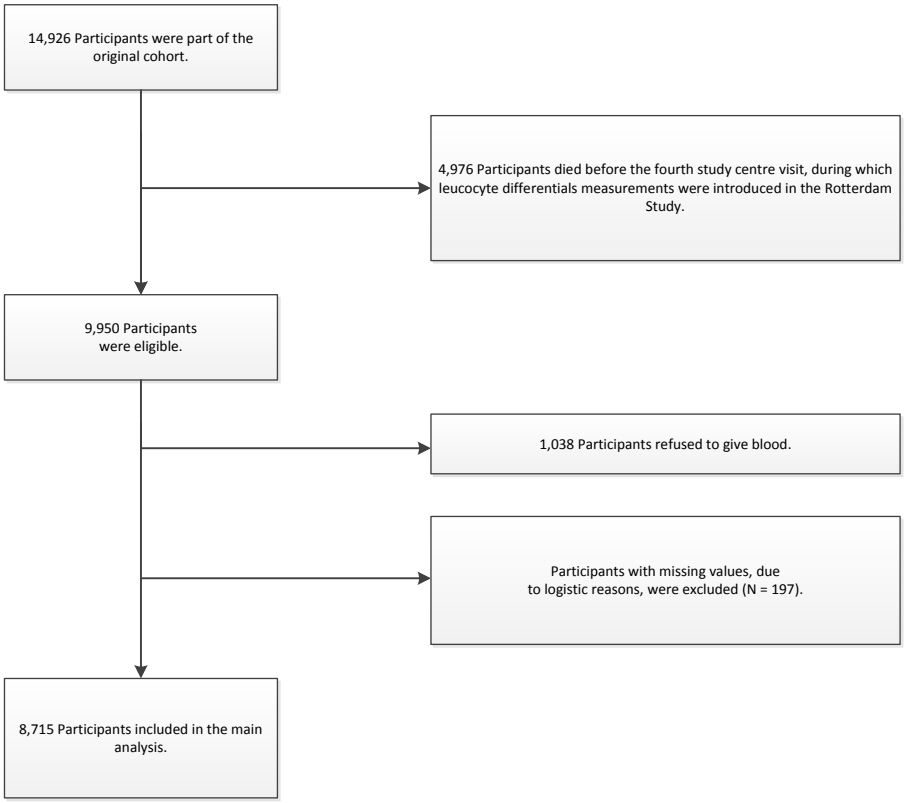
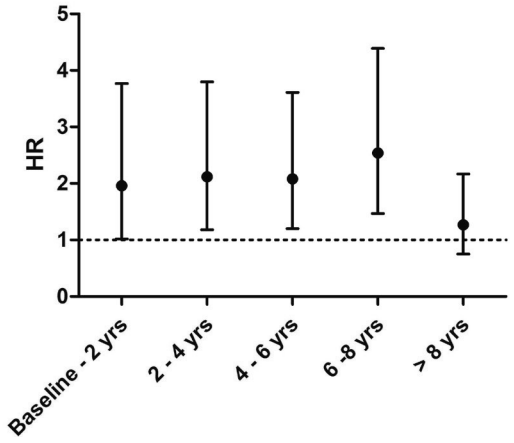
Supplementary Figure 1. Flowchart of the study population.

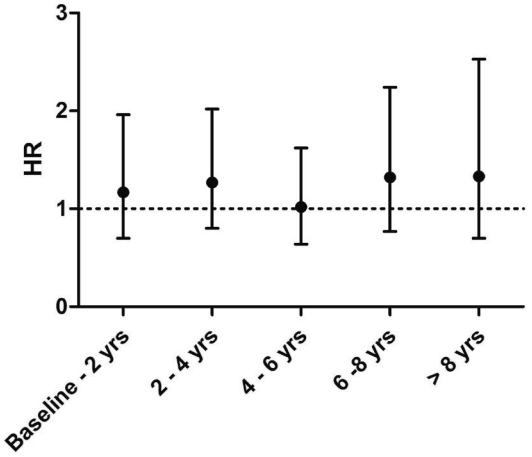


Supplementary Figure 2 A. Risk of NLR-related cardiovascular mortality



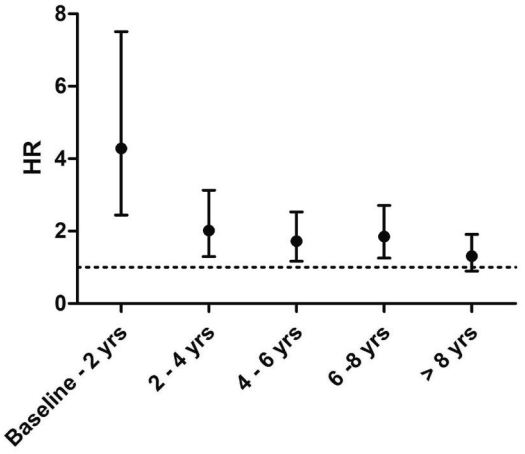
Adjusted for: sub-cohort, sex, age (in years), socio-economic status (high/intermediate/low), smoking status (current/former/never), BMI (body mass index, kg/m^2), prevalent type 2 diabetes mellitus and history of cardiovascular disease. Risk for each time stratum were for: baseline – 2 years (HR 1.96, 95% CI: 1.02 – 3.77), 2 – 4 years (HR 2.12, 95% CI: 1.18 – 3.80), 4 – 6 years (HR 2.08, 95% CI: 1.20 – 3.61), 6 – 8 years (HR 2.54, 95% CI: 1.47 – 4.39) and > 8 years (HR 1.27, 95% CI: 0.75 – 2.17).

Supplementary Figure 2 B. Risk of NLR-related cancer mortality



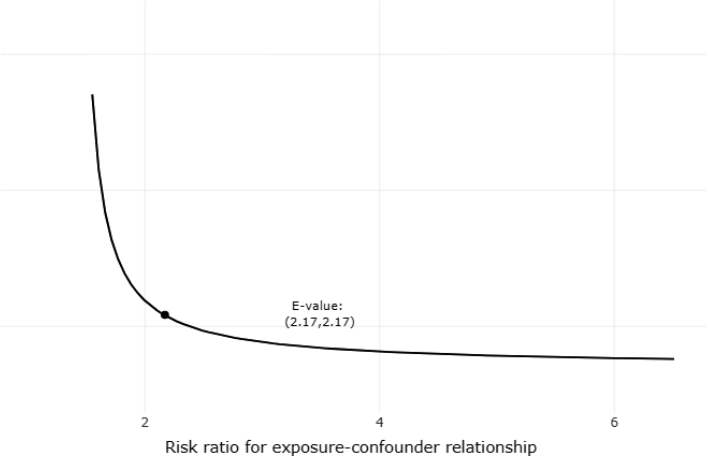
Adjusted for: sub-cohort, sex, age (in years), socio-economic status (high/intermediate/low), smoking status (current/former/never), BMI (body mass index, kg/m^2), prevalent type 2 diabetes mellitus and history of cancer. Risk for each time stratum were for: baseline – 2 years (HR 1.17, 95% CI: 0.70 – 1.96), 2 – 4 years (HR 1.27, 95% CI: 0.80 – 2.02), 4 – 6 years (HR 1.02, 95% CI: 0.64 – 1.62), 6 – 8 years (HR 1.32, 95% CI: 0.77 – 2.24) and > 8 years (HR 1.33, 95% CI: 0.70 – 2.53).

Supplementary Figure 2 C. Risk of NLR-related other mortality



Adjusted for: sub-cohort, sex, age (in years), socio-economic status (high/intermediate/low), smoking status (current/former/never), BMI (body mass index, kg/m^2), prevalent type 2 diabetes mellitus, history of cardiovascular disease and history of cancer. Risk for each time stratum were for: baseline – 2 years (HR 4.28, 95% CI: 2.44 – 7.51), 2 – 4 years (HR 2.02, 95% CI: 1.30 – 3.13), 4 – 6 years (HR 1.72, 95% CI: 1.17 – 2.53), 6 – 8 years (HR 1.85, 95% CI: 1.26 – 2.71) and > 8 years (HR 1.31, 95% CI: 0.90 – 1.91).

Supplementary Figure 3. E-value for the average hazard ratio for all-cause mortality.



Chapter 5

Erythrocyte sedimentation rate as an independent prognostic marker for mortality – a prospective population-based cohort study.

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Abstract

Background: A very high erythrocyte sedimentation rate (ESR) is usually an indication of underlying pathology. Additionally, a moderately elevated ESR may also be attributable to biological ageing itself. Whether the ESR is a prognostic factor for mortality, regardless of age, has been scarcely investigated. Therefore the objective was to analyze the association between elevated ESR levels and the risk of mortality in a prospective cohort of the general population.

Methods: We studied data from the Rotterdam Study (1990-2014). ESR levels were measured at baseline and individuals were followed until death or end of study. Associations between moderately (20-50mm/hour) and markedly (>50mm/h) elevated ESR levels and all-cause mortality, were assessed using multivariate Cox proportional hazard models.

Results: In total 5,226 participants were included, the mean age was 70.3 years. During a median follow-up time of 14.9 years 3,598 participants died (69%). After adjustment, both a moderately elevated ESR and a markedly elevated ESR were associated with a significantly higher risk of overall mortality (hazard ratio (HR) 1.23, 95% confidence interval (CI) 1.12-1.35 and HR 1.82, 95%CI 1.36-2.42, respectively). Although the ESR becomes higher with age , in a group aged above 75 years, without any comorbidities, an ESR>20mm/hour remained associated with a significantly increased risk of mortality (HR 1.29, 95%CI 1.01-1.64).

Conclusion: An elevated ESR is an independent prognostic factor for mortality. Despite the fact that ESR increases with age, it remains associated with an increased risk of mortality and warrants close follow-up.

Introduction

The erythrocyte sedimentation rate (ESR) measurement is a standardized, accurate, widely available, and inexpensive method to measure inflammation.[1] [2] The ESR is used globally, both by specialists in hospitals as well as by family doctors in primary care, as a routine test to screen for the presence of hidden inflammation, and to help in the diagnosis and follow-up of chronic diseases, such as rheumatoid arthritis, polymyalgia rheumatica, multiple myeloma, but also cardiovascular disease, and cancers. [2-4]

The ESR measures the aggregation of red blood cells. In the presence of increased levels of proteins like fibrinogen or globulins, erythrocytes aggregate easier, forming a rouleaux which settles at the lower end of the tube. When the red blood cell shape is taken into account, and by taking into account the level of hematocrit and hemoglobin, the ESR reflects the concentration of acute phase proteins and can be interpreted as a compound measure of inflammation.

Although a very high ESR is usually indicative of the presence of an underlying illness [4-7], the ESR is also subject to influences that are unrelated to disease. For instance, women tend to have higher ESR values than men; and increased body mass index (BMI) has also been associated with higher ESR values.[8,9]

Moreover, the ESR generally increases with age. This makes it difficult to interpret whether an increase of the ESR is due to hidden disease or 'part of the process of ageing'. It has been suggested that moderately elevated ESR levels can be attributed to biological ageing and can therefore be disregarded when this comes up during routine testing. [10] Yet, there is little evidence in literature to substantiate that interpretation. In contrast to the abundance of literature on C-reactive protein, there is only a limited number of studies that showed that the ESR is a risk factor for cancer and cardiovascular morbidity and mortality. [2,3] [11] Indeed, the general clinical experience is that an increased ESR at an older age is associated with an increased risk of mortality. But to this date there is only one study reporting on the independent association of ESR with an increased risk of mortality in older adults.[12]

Therefore, we set out to study the independent association of ESR levels and the risk of overall mortality in a population-based cohort. Our hypothesis was that even though ESR levels increase with age, they remain associated with an increased risk of mortality and therefore should not be disregarded.

Methods

Setting and study population

The objectives and design of the Rotterdam study have been extensively described earlier.[13,14] In brief, of the 10,275 persons aged 55 years and over, that were invited in 1989 7,983 (78%) participated and were followed ever since.

Detailed information was obtained at start of the study from all participants. They were interviewed at home by trained interviewers and had two subsequent visits at the research center where they underwent a physical examination, laboratory assessments, and imaging procedures. Follow-up examinations took place approximately every 3-4 years.

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

Assessment of ESR and other covariables

At baseline, blood was drawn directly into tubes (Vacutainer; BD Biosciences, Franklin Lakes, NJ), and the ESR was read after 60 minutes. The ESR was non-normally distributed and therefore log-transformed when analyzed as a continuous variable. We also categorized ESR into three groups: <20 mm/hour (reference), 20 – 50 mm/hour (moderately elevated) and >50 mm/hour (markedly elevated).

The following covariables were assessed as potential confounders: age, sex, socio-economic status (SES; according to education level; high/intermediate/low), smoking status (current/former/never), Body Mass Index (BMI in kg/m^2), prevalent type 2 Diabetes Mellitus (DM), history of cancer (based on pathology), history of cardiovascular disease including transient ischemic attacks (TIA), stroke (CVA), myocardial infarction (MI), coronary revascularization (percutaneous transluminal coronary angioplasty or coronary artery bypass grafting) and other cardiovascular disease (CVD), high-sensitivity C-reactive protein (CRP; Beckman Coulter, Fullerton, CA), hematocrit (I/l) and lastly presence of anaemia. DM was based on a fasting plasma glucose level of ≥ 7.0 mmol/L (≥ 126 mg/dL) or non-fasting plasma glucose level of ≥ 11 mmol/L (≥ 200 mg/dL) or use of blood glucose medication.[15] Anaemia was assessed as a haemoglobin concentration of less than 7.5 mmol/l for women or less than 8.5 mmol/l for men.[16]

Assessment of outcome

The main outcome of interest was all-cause mortality. The vital status of the participants was obtained regularly from the municipal population registry. Causes of death were assessed by reviewing information from the general practitioners' records or, in case of hospitalization, by discharge reports from the medical specialists. Causes of death were coded independently by two physicians using the ICD-10 and the ICPC-2. [17,18]

Statistical Analysis

Participants were followed from the day of inclusion in the study until the date of death or end of the study period (31st December 2013), whichever came first.

Difference in survival between individuals with a normal *versus* a moderately increased and markedly increased ESR were assessed by Kaplan – Meier curves and Log-rank tests. The associations between ESR and risk of overall mortality was analyzed with Cox proportional hazard models, using follow-up time as an underlying time scale. Proportional hazard assumptions were assessed visually. The results were reported as hazard ratios (HR) and 95% confidence intervals (CI).

All potential confounders, mentioned above, were assessed individually and were included in the multivariable model when they changed the point estimate by more than 10% or were considered clinically relevant (see **Supplementary Table 1**).

We additionally performed several sub-analyses. There is reason to believe different cut-off values should be used for males and females at different ages. Miller et al. suggested the following formulae for calculating the maximum normal erythrocyte sedimentation rate at a given age for men: (age in years / 2) and for women: (age in years + 10) / 2.[19] We assessed the risk of overall mortality using a cut-off following Millers' formula, in a multivariable Cox model. Additionally, we compared the risk classification according to Miller to the categories we created, using a two-by-two table.

Lastly, we assessed the risk of ESR in a highly selected group of healthy individuals older than 75 years of age, with a BMI of 18.5 – 29.9 kg/m^2, who were either former or never smokers and were free from diabetes, cardiovascular disease or cancer at baseline. Again, we used multivariable Cox regression.

Analyses were performed using IBM SPSS Statistics software (version 21) and R Version 3.3.2. All *P*-values were two-sided and were considered significant if $P < 0.05$.

Results

Population characteristics

Erythrocyte sedimentation rates were measured for 5,226 participants in the Rotterdam Study (65.5%). The mean age was 70.3 years and the majority were women (n = 3,232; 61.8%). The mean BMI was 26.3 kg/m2 , 10.8% was diagnosed with type 2 Diabetes Mellitus; 1,187 participants were current smokers (22.7%), 2,069 former smokers (36.9%) and 34.1% had never smoked at baseline. Further cohort characteristics are presented in **Table 1**. The median follow-up time was 14.9 years (SE 0.2), during which 3,599 participants died (68.9%).

Participants with a missing ESR value were significantly older, had higher socioeconomic status and were more likely to have diabetes. Participants with missing values of the ESR were less frequent smokers and were less likely to have a history of cardiovascular disease. No differences were found for sex, BMI, WHR, history of cancer and presence of anaemia (see **Supplementary Table** 2).

Erythrocyte sedimentation rate

The median ESR at baseline was 10 mm/hour (interquartile range (IQR): 6-18). The majority (n=3733; 71.4%) had an ESR less than 20 mm/hour, 27.0% had an ESR between 20-50 mm/h, and 95 individuals (1.6%) had an ESR of more than 50 mm/hour. Only two participants had an ESR higher than 100 mm/hour at baseline.
The median ESR for females was 12 mm/hour and significantly higher than the median ESR of 8 mm/hour for males (IQR respectively 8-19 mm/hour and 4-14 mm/hour; Mann-Whitney U Test: *P*-value <0.001). The median ESR was significantly higher for each age category: 8 mm/hour for 55-65 years, 10 mm/hour for 65-75 years and 14 mm/hour for >75 years (IQR respectively 5-14 mm/hour; 6-17 mm/hour and 8-23 mm/hour; Kruskal-Wallis Test: *P*-value <0.001).

The ESR was not significantly correlated with hemoglobin levels (*P*-value = 0.094) or hematocrit levels (*P*-value = 0.145), but was significantly correlated with CRP (R^2 = 0.17; *P*-value <0.001).

Main outcome

The overall survival was poorer for participants with a moderately elevated ESR (20-50 mm/hour) and markedly elevated ESR (>50 mm/hour), with a median survival of 10.3 and 7.0 years, respectively, compared to a median survival of 17.0 years for individuals with an ESR < 20 mm/hour (Logrank test: *P*-value <0.001, see **Figure 1**).

Table 1. Cohort characteristics at baseline.

Clinical Variable		**N (%)**
	Total	5226 (100)
Sex	Female	3232 (61.8)
	Male	1994 (38.2)
Age (years, (SD))		70.3 (9.2)
	55 – 64	1735 (33.2)
	65 – 75	1919 (36.7)
	>75	1572 (30.1)
Socio-economic status	High	324 (6.2)
	Intermediate	1245 (23.8)
	Low	3457 (66.2)
Smoking status	Current	1187 (22.7)
	Former	2069 (36.9)
	Never	1783 (34.1)
Diabetes Mellitus (prevalent)		565 (10.8)
Body mass index (kg/m^2, (SD))		26.3 (3.8)
	< 18.5	57 (1.1)
	18.5 – 24.9	1882 (36.0)
	25 – 29.9	2341 (44.8)
	≥ 30	770 (14.7)
Waist-to-hip ratio (meters, (SD))		0.90 (0.09)
Alcohol use (grams per day, (SD))		10.4 (15.4)
Anaemia*		1080 (20.7)
Hematocrit (l/l, (SD))		0.41 (0.04)
History of Cardiovascular Disease		430 (8.2)
History of Cancer		22 (0.4)
CRP (mg/mL, (SD))		3.3 (6.0)
ESR (mm/hour, (Median, IQR))		10 (6 – 18)
ESR (mm/hour)	<20	3733 (71.4)
	20 – 50	1410 (27.0)
	>50	83 (1.6)

Data are presented in numbers with percentages in brackets unless stated otherwise. Numbers do not add up to 100% due to missing values. CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, SD: standard deviation.

* Anaemia was assessed as a haemoglobin concentration of less than 7.5 mmol/l for women or less than 8.5 mmol/l for men.

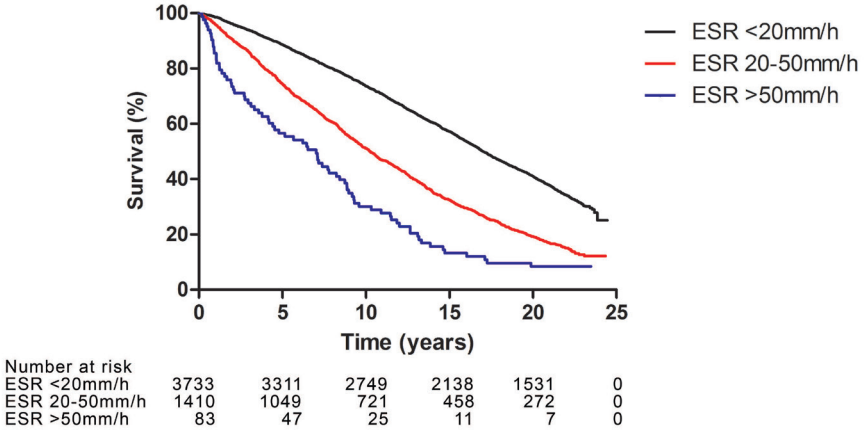

Figure 1. Kaplan-Meier Curves for each category of the erythrocyte sedimentation rate (ESR).

In the crude analysis, both an ESR of 20-50 mm/hour and an ESR > 50 mm/hour were associated with a significantly increased risk of mortality with a HR of 1.91 (95%CI: 1.78 – 2.05) and a HR of 3.23 (95%CI: 2.57 – 4.06), respectively (see **Table 2**). After adjustment for age, sex, SES, BMI, WHR, smoking status, prevalent DM, hematocrit level, history of CVD, and history of cancer the association remained, with a statistically significantly increased risk of 23% for individuals with a moderately elevated ESR and 82% for individuals with a markedly increased ESR (HR 1.23, 95% CI 1.04-1.45 and HR 2.02, 95% CI 1.18-3.45, respectively; see **Table 2**) compared with a ESR below 20 mm/hour. Also, after additional adjustment for CRP the associations remained robust (see **Table 2**).

To assess whether the found effects were in fact caused by any sub-clinically present disease, we excluded all individuals with a follow-up of less than 2 and 5 years and repeated the analysis. The association remained (see **Supplementary Table 3**).

Sub-analyses

When the ESR normal value according to Millers formula was used, individuals with an increased ESR (n= 186, 3.6%) had a poorer survival (Logrank test: *P*-value <0.001) of 8.4 years of survival *versus* 15.3 years of survival. After adjustment for the above mentioned variables, an increased ESR according to Millers' formula was an independent marker of mortality with an increased risk of 62% (HR 1.62, 95% CI: 1.32 – 1.98). We compared the classification according to Millers' formula with the classification when using the following categories for ESR: <20mm/h, 20-50mm/h and >50mm/h (see **Supplementary Table 4**). All individuals with an ESR>50mm/h were classified as having an increased ESR according

Table 2. Cox proportional hazard regression for all-cause mortality.

Model	Events/cohort	ESR	HR	Lower 95% CI	Upper 95% CI
Crude	3749/5226	Logtransformed	1.36	1.30	1.42
		<20 mm/hour	reference	-	-
		20 – 50 mm/hour	1.91	1.78	2.05
		>50 mm/hour	3.23	2.57	4.06
Sex and age adjusted	3749/5226	Logtransformed	1.19	1.14	1.25
		<20 mm/hour	reference	-	-
		20 – 50 mm/hour	1.26	1.17	1.35
		>50 mm/hour	2.03	1.62	2.56
Multivariable I*	2383/3475	Logtransformed	1.15	1.09	1.21
		<20 mm/hour	reference	-	-
		20 – 50 mm/hour	1.23	1.12	1.35
		>50 mm/hour	1.89	1.38	2.60
Multivariable II†	2224/3244	Logtransformed	1.09	1.03	1.16
		<20 mm/hour	reference	-	-
		20 – 50 mm/hour	1.16	1.06	1.28
		>50 mm/hour	1.69	1.20	2.38

*Adjusted for: sex, age in years, SES (socio-economics status: high/intermediate/low), smoking status (current/former/never), BMI (body mass index: <18.5 kg/m^2, 18.5-24.9 kg/m^2, 25.0 – 29.9 kg/m^2, ≥30.0 kg/m^2), WHR (waist-to-hip-ratio), Type-2 diabetes, history of cancer, history of cardiovascular disease and hematocrit. Alcohol use and anemia did not contribute significantly to the model nor changed the point estimate of ESR by more than 10% and were therefore not included in the multivariable model.

†Multivariable model I, additionally adjusted for CRP.

ESR: erythrocyte sedimentation rate, HR: hazard ratio, 95% CI: 95% confidence interval, CRP: C-reactive protein.

Millers' formula and thus as having an increased risk. However for individuals with an ESR 20-50mm/h the majority (93%) was not classified as having an increased ESR in agreement with Millers' formula.

Lastly, we explored whether for healthy individuals with an age above 75 years, ESR remained an independent predictor of mortality. We selected a group of elderly with a BMI between 18.5 – 30 km/m^2, who were former or never smokers, had no prevalent DM, and were free from cardiovascular disease or cancer diagnosis at baseline. In this highly selected healthy group of elderly, an ESR >20mm/hour was significantly associated with increased risk of mortality (see **Table 3**). Healthy individuals with an ESR above 20 mm/hour (n=139, 21.8%) had a poorer survival of 7.5 years *versus* 8.8 for those with a ESR < 20 mm/hour (Logrank test: *P*-value = 0.064). After adjustment for the above mentioned variables, an ESR > 20 mm/hour was an independent predictor of mortality with an increased risk of 29% (HR 1.29, 95% CI: 1.01-1.64) *versus* an ESR < 20 mm/hour.

Table 3. ESR as a risk factor for all-cause mortality, also in the healthy elderly.

			HR*	**Lower 95% CI**	**Upper 95% CI**
> 75 with comorbidities	$ESR_{logtransformed}$		1.33	1.16	1.52
	$ESR_{categorical}$	≤20 mm/h	reference		
		>20 mm/h	1.59	1.28	1.98
> 75 years and healthy†	$ESR_{logtransformed}$		1.13	0.98	1.31
	$ESR_{categorical}$	≤20 mm/h	reference		
		>20 mm/h	1.29	1.01	1.64

* Adjusted for: sex, age in years, SES (socio-economics status: high/intermediate/low), smoking status (current/former/never), BMI (body mass index: <18.5 kg/m^2, 18.5-24.9 kg/m^2, 25.0 – 29.9 kg/m^2, ≥30.0 kg/m^2), WHR (waist-to-hip-ratio), Type-2 diabetes, history of cancer, history of cardiovascular disease and hematocrit.
† Healthy elderly was defined as those with a body mass index of 18.5-29.9 kg/m^2, never or former smoker, no Type-2 diabetes and no prevalent cardiovascular disease or cancer at baseline.
ESR: erythrocyte sedimentation rate, HR: hazard ratio, 95% CI: 95% confidence interval.

Discussion

In this study we showed that both a moderately increased, as well as a markedly increased ESR, are associated with a significantly higher risk of overall mortality. These results follow from a large population-based prospective cohort of community dwelling elderly with a follow-up period of almost 25 years. We found that the associations were most attenuated by sex and age and to a smaller extent by smoking and comorbidities such as diabetes, cardiovascular disease or cancer. However the relationship remained robust after adjustment for these and other confounders, such as hematocrit and CRP. We additionally

presented that, although ESR levels are influenced by age, even in a highly selected group of individuals aged >75 years without comorbidities, a moderately elevated ESR remains a risk factor for mortality.

As discussed previously, the ESR can be used as a compound measure for systemic inflammation. Despite this fact, the ESR has been scarcely studied both with respect to morbidity [3,20 21] and mortality[2,22 23 24], especially in a population-based setting[12]. An increased risk of cardiovascular mortality was demonstrated earlier in patients with cardiovascular disease.[23 24] More recently, the risk for incident colorectal cancer and the risk for overall mortality in the general population were studied, and ESR was found to be an independent risk factor for both.[3,12] For the latter however this study was limited in size and in its analysis as mortality was not the main outcome.

In addition to continuous analyses, we explored clinically relevant cut-off points. In the crude analysis, we found that individuals with a moderately and markedly elevated have a 2-fold and 3-fold higher risk for mortality, respectively. After adjustment for sex and age an ESR >50mm/hour remained associated with a 2-fold increased risk. This is a specifically important finding for general practitioners, for whom additional diagnostic tools are usually limited to blood tests, because it is an easy to use measurement to identify those patients who may be at highest risk of dying.

It has been suggested that using sex- and age specific cut-off values, with higher cut-off values for women and a higher age, would be more appropriate.[19] In this cohort indeed the median ESR level was significantly higher in women than in men and significantly higher for the higher age categories, but there was no statistical interaction with sex nor with age. We explored the risk of overall mortality using cut-off values following Millers' formula. Results remained comparable, but importantly we found that misclassification was present. Whereas all individuals with an ESR > 50 mm/hour were also classified as having an increased ESR using Millers' formula, the majority of individuals with an ESR between the 20 and 50 mm/hour were misclassified as having an age-appropriate and thus 'normal ESR'. But in our analyses also those with an ESR between 20-50 mm/hour are at an increased risk of mortality.

These findings are in line with previous research on the cut-off levels for hemoglobin in the elderly. [25] Although anaemia is more prevalent in oldest old patients, it is still associated with an increased risk of mortality, justifying the use of the same cut-off levels in young and old patients.

Additionally, our results also challenge the thought that a moderately increased ESR in elderly, that cannot be attributed to comorbidities, can be safely discarded. We showed that a moderately increased ESR in group of elderly with a normal BMI, who are not currently smoking and are free of diabetes, cardiovascular disease and cancer still have an increased risk of dying, compared to those with an ESR <20 mm/hour. These patients should therefore receive follow-up.

Strengths and limitations

This is the first study to date, that prospectively examines the association between an increased ESR and risk of mortality in the elderly. Additionally, we collected detailed information on illness and risk factors for chronic diseases, that allowed for proper adjustment of the relationship between ESR and mortality. We showed that, although the association is attenuated by several confounders, it remains a robust prognostic marker.

There are a few limitations that warrant mentioning. The first one is that an ESR level was measured for only 65.5% of the original cohort. The major reason for these missings, was that since 1993, the ESR measurement was not performed any longer for logistic reasons. Therefore the missing values are completely at random. Additionally, a missing ESR measurement was not associated with mortality, therefore we do not believe that if we would have had these random-missing measurements the outcomes of the study would be different. Second, we only measured ESR once. Therefore, we cannot verify whether some of the increased ESR levels are in fact limited to a short period due to a transient illness. However, this specific type of misclassification would have resulted in an underestimation of the risk of mortality because the group of individuals who have persistently increased ESR levels is diluted by those with a transient ESR elevation with little or no increased risk.

In conclusion the ESR is a robust marker for overall mortality, even when it is only moderately increased and regardless of age. Therefore, it is justified to use the same cut-off values for young and old patients and an increased ESR at an older age should not be disregarded but instead warrants further follow-up.

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Supplementary Table 1. Univariable Cox proportional hazard regression for overall mortality.

Variable		**Univariable analysis**		
		HR	Lower 95% CI	Upper 95% CI
Female		0.79	0.74	0.84
Age		1.12	1.115	1.124
SES	High	reference		
	Intermediate	1.15	0.99	1.34
	Low	1.37	1.18	1.58
Smoking	Current	ref		
	Former	0.82	0.75	0.89
	Never	0.85	0.78	0.93
DM		1.19	1.13	1.25
BMI		0.99	0.98	1.00
BMI	< 18.5	2.24	1.69	2.96
	18.5 – 25.0	reference		
	25.0 – 30.0	0.97	0.91	1.05
	> 30.0	1.00	0.91	1.11
WHR		12.09	8.47	17.26
Alcohol use		1.002	1.000	1.005
Anaemia		0.93	0.85	1.00
Haemotocrit		0.64	0.30	1.38
History CVD		2.04	1.83	2.27
History of Cancer		1.66	1.03	2.68
$CRP_{logtransformed}$		1.31	1.27	1.36
$ESR_{logtransformed}$		1.36	1.30	1.42
$ESR_{categorical}$	< 20 mm/h	reference		
	20 – 50 mm/h	1.91	1.78	2.05
	>50 mm/h	3.23	2.57	4.06

Age in years, BMI (Body Mass Index in kg/m^2), WHR (Waist Hip Ratio in meters), Alcohol in average intake per day in grams, Anaemia (for males Hb< 8.5 mmol/l and females Hb<7.5 mmol/l), DM (Diabetes Mellitus), CVD (cardiovascular disease), CRP (C-reactive protein), ESR (Erythrocyte Sedimentation Rate in mm/hour), HR (hazard ratio), CI (confidence interval)

Supplementary Table 2. Population characteristics for participants with and without an ESR measurement.

Clinical Variable		ESR available N (%)	ESR not available N (%)	*P*-value
	Total	5226 (100)	2757 (100)	
Sex	Female	3232 (61.8)	1646 (59.7)	0.062
	Male	1994 (38.2)	1111 (40.3)	
Age (years, (SD))		70.3 (9.2)	71.2 (10.8)	<0.001
	55 – 64	1735 (33.2)	981 (35.6)	<0.001
	65 – 75	1919 (36.7)	788 (28.6)	
	>75	1572 (30.1)	988 (35.8)	
Socio-economic status	High	324 (6.2)	294 (10.7)	<0.001
	Intermediate	1245 (23.8)	688 (25.0)	
	Low	3457 (66.2)	1561 (56.6)	
Smoking status	Current	1187 (22.7)	538 (19.5)	0.002
	Former	2069 (36.9)	1038 (37.6)	
	Never	1783 (34.1)	1011 (36.7)	
Diabetes Mellitus		565 (10.8)	318 (11.5)	0.038
BMI (kg/m^2, (SD))		26.3 (3.8)	26.2 (3.6)	0.551
	< 18.5	57 (1.1)	20 (0.7)	0.173
	18.5 – 24.9	1882 (36.0)	679 (24.6)	
	25 – 29.9	2341 (44.8)	910 (33.0)	
	≥ 30	770 (14.7)	251 (9.1)	
WHR (m, (SD))		0.90 (0.09)	0.91 (0.09)	0.121
Alcohol use (gr/day, (SD))		10.4 (15.4)	10.3 (14.5)	0.912
Anaemia*		1080 (20.7)	603 (21.9)	0.330
Hematocrit (l/l, (SD))		0.41 (0.04)	0.41 (0.04)	0.288
History of CVD		430 (8.2)	178 (6.5)	0.015
History of Cancer		22 (0.4)	16 (0.6)	0.325

Differences between individuals with and without an ESR measurement were tested using Students' T-tests for normally distributed continuous variables and χ^2-tests for categorical variables.

ESR = erythrocyte sedimentation rate, BMI = Body Mass Index, WHR = waist-to-hip ratio, CVD = cardiovascular disease, SD = standard deviation.

Supplementary Table 3. Multivariable Cox regression for all-cause mortality for individuals with more than 2 and 5 years of follow-up.

Follow-up			HR*	Lower 95% CI	Upper 95% CI
> 2 years	$ESR_{logtransformed}$		1.13	1.06	1.19
	$ESR_{categorical}$	< 20 mm/h	reference		
		20 – 50 mm/h	1.22	1.11	1.34
		> 50 mm/h	1.76	1.24	2.49
> 5 years	$ESR_{logtransformed}$		1.11	1.04	1.18
	$ESR_{categorical}$	< 20 mm/h	reference		
		20 – 50 mm/h	1.20	1.09	1.33
		> 50 mm/h	1.83	1.24	2.70

*Adjusted for: sex, age in years, SES (socio-economics status: high/intermediate/low), smoking status (current/former/never), BMI (body mass index: <18.5 kg/m^2, 18.5-24.9 kg/m^2, 25.0 – 29.9 kg/m^2, ≥30.0 kg/m^2), WHR (waist-to-hip-ratio in meters), type-2 diabetes, history of cancer, history of cardiovascular disease and hematocrit.

Supplementary Table 4. Two-by-two-table for the comparison of classification of an increased ESR according to our categories versus Millers' Formula.

		ESR increased according to Millers' Formula		Total
		No	Yes	
ESR category	<20mm/h	3733	0	3733
	20-50mm/h	1307	103	1410
	>50mm/h	0	83	83
Total		5040	186	5226

Chapter 6

The systemic immune-inflammation index is associated with an increased risk of incident cancer - results from the Rotterdam Study.

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Abstract

Background: Several studies found that the systemic immune-inflammation index (SII) is a prognostic factor for mortality in patients with solid tumors. It is unknown whether an increased SII in generally healthy individuals reflects a risk for developing cancer. Our objective was to investigate the association between the SII and incident cancers in a prospective cohort study.

Methods: Data were obtained from the Rotterdam Study; a population-based study of individuals aged ≥45 years, between 2002 and 2013. The SII at baseline was calculated from absolute blood counts. The association between the SII and the risk of any solid incident cancer during follow-up was assessed using Cox proportional hazard models. Individuals with a prior cancer diagnosis were excluded.

Results: Data of 8,024 individuals were included in the analyses. The mean age at baseline was 65.6 years (SD 10.5 years) and the majority were women. During a maximum follow-up period of 10.7 years, 733 individuals were diagnosed with cancer. A higher SII at baseline was associated with a 30% higher risk of developing a solid cancer (HR of 1.30 (95% CI; 1.11 – 1.53)), after adjustment for age, sex, socio-economic status, smoking, BMI and type 2 diabetes. The absolute cumulative 10-year cancer risk increased from 9.7% in the lowest quartile of SII to 14.7% in the highest quartile (*P*-value = 0.009). The risk of developing cancer was persistent over time and increased for individuals with longest follow-up.

Conclusion: A high SII is a strong and independent risk indicator for developing a solid cancer.

Introduction

In 1863, Virchow observed the presence of leukocytes in neoplastic tissues and hypothesized an association between inflammation and cancer. [1] Since then, various theories regarding this presumed association have been proposed. [2-5] One theory suggests that low-grade, chronic inflammation increases the risk of cancer. [3] For example, a *Helicobacter Pylori* infection is associated with gastric cancer, inflammatory bowel disease with colorectal cancer, and tobacco smoke, in addition to being carcinogenetic, can induce chronic inflammation and is associated with lung cancer. [3,6] Alternatively, inflammation is considered a consequence, rather than the cause, of cancer. [1]

Inflammatory markers in blood can be used as biomarkers to study these hypotheses. Well-known inflammatory markers include C-reactive protein, erythrocyte sedimentation rate and white blood cell count. [7-11] A relatively novel inflammatory marker in this respect is the systemic immune-inflammation index (SII). [12]

It is an index that incorporates the absolute blood counts of neutrophils, lymphocytes as well as platelets, by multiplying the platelet count by the ratio of neutrophil and lymphocyte counts. Several studies found that the SII is a prognostic factor in patients with solid cancers, such as hepatocellular carcinoma, colorectal, and pancreatic cancer. [12-14] So far, it is unknown whether an increased SII also is a marker for developing incident cancer in healthy individuals.

We hypothesized that when inflammatory cells play a role in the etiology of cancer, individuals with higher levels of inflammation, as measured by the SII, over a longer period of time are at a higher risk to develop cancer. Therefore the objective of this study was to assess the relationship between SII levels at baseline and the subsequent risk of developing a solid cancer in a prospective, population-based cohort.

Methods

Study Setting

The study was embedded in the Rotterdam Study, an ongoing prospective cohort study in community-dwelling elderly in the Ommoord suburb of the city of Rotterdam in the Netherlands. The rationale and design have been previously been described. [15] Briefly, in 1989, inhabitants aged 55 years and older were invited to participate. The original cohort was enrolled between 1989 and 1993 of whom 7,983 participated (78%). A second cohort of 3,011 persons (67% participation) was enrolled between 2000 and 2001. In 2006, a third cohort with 3,932 persons of 45 years and older were enrolled (65% participated). This resulted in an overall study population of 14,926 individuals aged 45 years and above.

Study Population

Baseline values of the SII were measured at the earliest study center visit at which a leukocyte differential count was available: the fourth visit of the first cohort (January 2002 – July 2004; n = 3,550), the second visit of the second cohort (July 2004 – December 2005; n = 2,468), and the first visit of the third cohort (February 2006 – December 2008; n = 3,932) (see **Supplementary Figure 1**, [16]). Data of individuals with missing granulocyte, lymphocyte or platelet counts or of individuals with a diagnosis of cancer (except non-melanoma skin cancer) prior to the initial blood count at baseline were excluded (n=687, see **Figure 1**).

Assessment of the systemic immune-inflammation index (SII)

Fasting blood samples were collected at the study center and full blood count measurements were performed immediately after blood draw. These measurements included absolute counts of granulocytes, lymphocytes, and platelets and were performed using the COULTER® Ac·T diff2™ Hematology Analyzer (Beckman Coulter, San Diego, California, USA).

The systemic immune-inflammation index (SII) was calculated from the platelet (P; $x10^9$/Liter), granulocyte, as a proxy for neutrophils, (N; $x10^9$/Liter), and lymphocyte (L; $x10^9$/Liter) blood counts, using the following formula: SII = P x N/L. [12] Both the neutrophil-to-lymphocyte-ratio (NLR = N/L) and the platelet-to-lymphocyte ratio (PLR = P/L) were also calculated.

Collection of other variables

The following variables were considered as potential confounding factors: age, sex, socio-economic status (high/intermediate/low), smoking status (current/former/never), and body mass index (BMI; kg/m^2). Individual characteristics were determined at baseline by interview or at the study center. Status on prevalent type 2 diabetes was ascertained from general practitioners' records (including laboratory glucose measurements), hospital discharge letters, and serum glucose measurements at the study center. Diabetes was defined, according to the WHO guidelines, as a fasting glucose ≥ 7.0 mmol/Liter or use of glucose – lowering medication. [17]

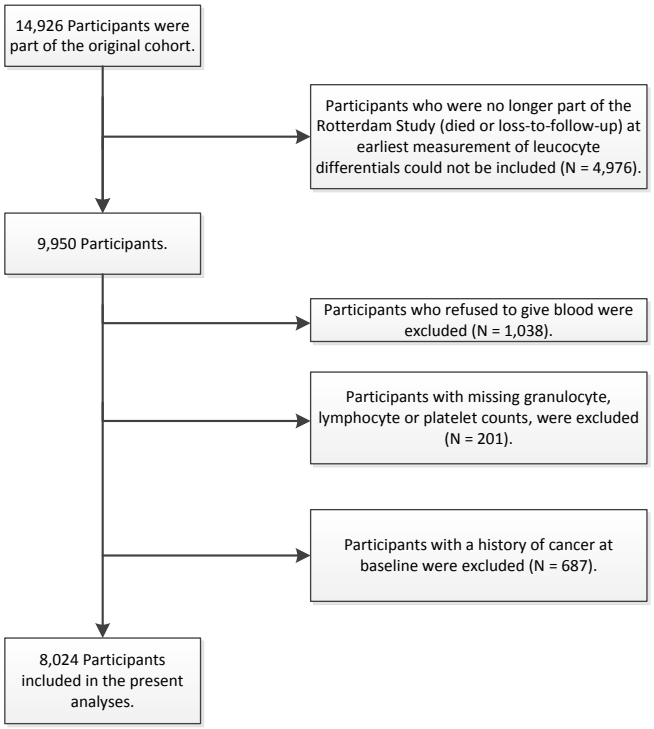
Assessment of outcome

The outcome of interest was the incident diagnosis of cancer. Cancer cases were identified from general practitioners' medical records (including hospital discharge letters), the Dutch Hospital Data registry and regional histopathology and cytopathology registries. Cases were coded independently by two physicians and classified according to the International Statistical Classification of Diseases, 10th revision (ICD-10) and the

International Classification of Primary Care, 2nd edition (ICPC-2). [18,19] Information on cancer was available up till 1st January 2013. Only pathologically verified cases were used in the analyses. Incident solid cancers were defined as any primary malignant tumor, except non-melanoma skin cancers or hematological malignancies.

Dates of death were obtained through the Netherlands Personal Records Database (BRP) and the causes of death were obtained from of general practitioners' records or hospital discharge letters and coded similarly as morbidity. [18,19]

Figure 1. Flowchart of the study population inclusion.



Statistical Analysis

We explored all three biomarkers (NLR, PLR and SII) and compared models including the three biomarkers using the Akaike Information Criterion (see **Supplementary Table 1**).[20] We found that the SII performed the best, therefore only the results comprising the SII were reported. Participants were divided into quartiles based on the SII established at baseline. Differences between the quartiles were assessed with ANOVAs for normally distributed continuous variables and with -tests for categorical variables. We estimated the absolute risk of being diagnosed with a solid cancer for each quartile of the SII using the cumulative incidence. Differences across the strata were tested using Gray's tests. [21-23]

The relationship between the SII level at baseline and the risk of any solid cancer during follow-up was assessed using Cox proportional hazard models (separate analyses were performed for breast, prostate, colorectal, lung, and bladder cancer). For each individual, follow-up was defined in years, from the baseline date as described above, until the date of cancer diagnosis, death or end of study period (1st of January 2013), whichever came first.

The results are reported as hazard ratios (HR) and 95% confidence intervals (CI). The SII was log-transformed prior to being entered in any of the analyses. The proportional hazard assumption was assessed for all variables, using the Kaplan-Meier estimates for the categorical variables and the Schoenfeld's residuals for the continuous variables. [24]

All analyses were adjusted for previously mentioned cancer risk factors, i.e. age, sex, SES, smoking status, BMI and diabetes. Variables were added to the crude model in a stepwise approach when: a variable changed the effect estimate by more than 10% or when a variable was considered clinically relevant. [25] Effect modification was assessed for smoking and BMI by adding an interaction variable to the model and was considered statistically significant at a P-value < 0.10.

First we analyzed the SII as a continuous variable. Then, to assess whether there was a quartile-effect relationship, we stratified the SII into quartiles, in which the lowest one was taken as a reference category.

To explore whether the SII could be a marker of yet undetected disease we repeated the analysis only assessing the risk of cancer in the first 6 months of follow-up. To investigate whether the overall effect was not solely due to an inflammatory response to undetected cancers, and in fact a case of reverse causality, we additionally performed an analysis in which data of individuals with a follow-up of less than 6 months, 2 years, 5 and 8 years, respectively, were subsequently excluded.

Statistical significance of associations was accepted at a P-value < 0.05. All analyses were performed using SPSS software (Version 21.0) and SAS (Version 9.4). [26]

Results

General characteristics of the study population

Data of 8,024 individuals were included in the analyses (see **Figure 1**). The mean age at baseline was 65.6 years (standard deviation (SD) 10.5 years) and 57.3% were women (n=4,597). The mean BMI was 27.1 kg/m² (SD 4.1), 20.4% was a current smoker (N = 1,632), 48.6% a former smoker (3,897) and 10.9% had diabetes at baseline (N = 872). The median SII was 455 (IQR: 339 – 618). Population characteristics for each quartile of the SII can be found in **Table 1**.

The total follow-up was 53,582 person years with a maximum of 10.7 years per person; for more than three quarters of the participants the follow-up period was at least 5 years. Completeness of follow-up at the 1st of January 2013 was 98.7%.

Table 1. General cohort characteristics at baseline for each quartile of the SII.

Characteristic		**Systemic immune-inflammation index**				
		Q1	**Q2**	**Q3**	**Q4**	***P*-value**
		< 339	339 – 455	456 – 618	> 618	
	Total	N (%)	N (%)	N (%)	N (%)	
		2,006	2,006	2,006	2,006	
Sex	Male	915 (45.6)	854 (42.6)	837 (41.7)	821 (40.9)	<0.001
	Female	1,091 (54.4)	1,152 (57.4)	1,169 (58.3)	1,185 (59.1)	
Age (in years)	Mean (SD)	65.0 (9.9)	64.9 (10.2)	65.5 (10.6)	67.2 (11.0)	<0.001
Smoking *	Current	346 (17.2)	388 (19.3)	440 (21.9)	458 (22.8)	<0.001
	Former	987 (49.2)	1,001 (49.9)	937 (46.7)	972 (48.5)	
	Never	649 (32.4)	595 (29.7)	600 (29.9)	547 (27.3)	
SES *	High	392 (19.5)	413 (20.6)	387 (19.3)	339 (16.9)	0.009
	Intermediate	830 (41.4)	854 (42.6)	830 (41.4)	805 (40.1)	
	Low	758 (37.8)	718 (35.8)	764 (38.1)	827 (41.2)	
BMI (in kg/m²) *	Mean (SD)	27.0 (3.7)	27.2 (4.1)	27.2 (4.2)	27.1 (4.5)	0.133
DM status	Yes	187 (9.3)	208 (10.4)	220 (11.0)	257 (12.8)	0.004
	No	1,819 (90.7)	1,798 (89.6)	1,786 (89.0)	1,749 (87.2)	

*Unknown: SES (N = 107, 1.3%), Smoking (N = 104, 1.3%), BMI (N = 146, 1.8%) SES: socio-economic status, BMI: body mass index, DM: Diabetes Mellitus

Development of a solid cancer

In total, 733 individuals (9.1%) developed a solid cancer during follow-up. The most frequent cancers were: colorectal (N = 123, 16.8%), prostate (N = 112, 15.3%), breast (N = 99, 13.5%), lung (N = 95, 13.0%), and bladder cancer (N = 83, 11.3%). Other solid cancers included: esophagus, kidney, pancreas, melanoma, and gastric cancer.

A higher SII at baseline was associated with a 43% increased risk of a solid cancer in the univariable analysis (HR: 1.43; 95%CI 1.22 – 1.67) and a 30% increased risk when adjusted for cancer risk factors mentioned above (HR 1.30; 95% CI: 1.11 – 1.53) (see **Tables 2 and 3**). The effect of the SII was not modified by either smoking or BMI.

In the stratified analysis, the risk was higher in each subsequent quartile, with a significantly higher risk in the fourth quartile in comparison to the lowest quartile (HR: 1.39, 95% CI; 1.12 – 1.72), with a significant trend over the quartiles (*P*-value = 0.002, see **Table 3**).

The absolute 5- and 10- year risk of being diagnosed with a solid cancer were 5.4% and 9.7% in the lowest quartile compared to 7.2% and 14.7% in the highest quartile, respectively (see **Figure 2**).

Table 2. Univariate Cox Proportional Hazard regression for the association between baseline characteristics and diagnosis of a solid cancer.

Clinical Variable		**Univariable analysis**		
		HR	Lower 95% CI	Upper 95% CI
Cohort	RS-I	reference		
	RS-II	0.92	0.78	1.09
	RS-III	0.43	0.35	0.53
Female		0.58	0.50	0.67
Age (in years)		1.03	1.03	1.04
SES	High	reference		
	Intermediate	1.07	0.86	1.32
	Low	1.15	0.93	1.42
Smoking	Never	reference		
	Former	1.52	1.27	1.83
	Current	1.71	1.38	2.13
DM		1.62	1.33	1.98
BMI (in kg/m^2)		1.01	0.99	1.03
SII	Logarithm	1.43	1.22	1.67

SES: socio-economic status, DM: type II diabetes status, BMI: body mass index, SII: systemic immune-inflammation index, HR: hazard ratio, CI: confidence intervals

Table 3. Multivariable Cox Proportional Hazard regression for the association between baseline levels of the SII with development of a solid tumor.

SII	Total Follow-Up		Follow-Up > 6 months		Follow-Up > 2 years		Follow-Up > 5 years		Follow-Up > 8 years	
	HR*	95% CI	HR*	95% CI	HR*	95% CI	HR*	95% CI	HR*	95% CI
Q1	Reference		Reference		Reference		Reference		Reference	
Q2	1.13	0.91 – 1.42	1.11	0.88 – 1.40	1.12	0.86 – 1.45	1.23	0.81 – 1.88	1.19	0.40 – 3.55
Q3	1.23	0.98 – 1.53	1.19	0.95 – 1.49	1.26	0.97 – 1.62	1.56	1.05 – 2.34	1.73	0.64 – 4.63
Q4	1.39	1.12 – 1.72	1.33	1.07 – 1.66	1.37	1.07 – 1.77	1.82	1.22 – 2.71	2.92	1.15 – 7.36
Logarithm	1.30	1.11 – 1.53	1.26	1.07 – 1.50	1.27	1.05 – 1.54	1.48	1.10 – 1.99	2.20	1.12 – 4.32
P-value for trend	0.002		0.010		0.009		0.001		0.009	

Number of patients/cohort: 692/7703 for the total follow-up period, 646/7643 for follow-up > 6 months, 508/7406 for follow-up > 2 years, 213/6014 for follow-up > 5 years and 44/2360 for follow-up >8 years.

*Adjusted for: cohort, sex, age (years), socio-economic status (high/intermediate/low) , smoking status (current/former/never), BMI (body mass index, kg/m^2) and Type II diabetes status.

SII: Systemic Immune-Inflammation Index

The risk of developing a solid cancer after a high baseline SII was significantly higher within the first 6 months after baseline, with a HR of 2.00 (95% CI; 1.09 – 3.67). The risk was persistent over time and increased for individuals with longer follow-up times (see **Table 3**).

Next, we assessed the effects for the five major cancers in this population (colorectal, prostate, breast,lung, and bladder cancer). These effects were similar for colorectal, prostate lung, and bladder cancer, but we found null results for breast cancer (see **Supplementary Figure 2**).

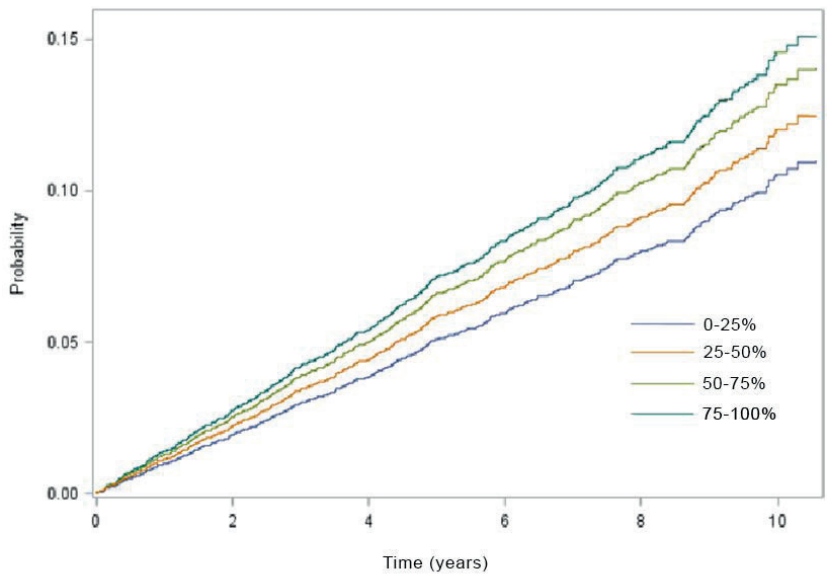


Figure 2. Absolute risk of being diagnosed with a solid cancer for each quartile of the SII.

Discussion

The association between inflammation and cancer is well known, and only partly understood as a result of its complex nature. [2-4] On the one hand, inflammation is thought to induce cancer, but on the other hand it may also be secondary to a systemic inflammatory response to yet-undetected tumor and accumulated DNA-damage. In both occasions, the products of inflammatory processes can be considered as potential biomarkers. [2-5,9] These markers have a prognostic and potentially also a predictive value in solid cancers. [27,28]

To our best knowledge, this is the first study on the etiological association between the SII and incident cancers in the general population. The SII is a relatively new composite measure of the neutrophil, lymphocyte, and platelet counts in the peripheral blood. [12]

Neutrophils were traditionally considered innocent bystanders in the cancer setting. More recently it has been assumed, however, that neutrophils may be important in tumor initiation, progression, and metastasis. [29,30] Pro-metastatic effects of platelets are attributed to the adhesion of platelets to tumor cells, thereby providing a shield protecting against cell death, but also to platelet-derived factors that enable cells to migrate from the blood stream into visceral organs. [31,32] Lymphocytes, on the other hand, are thought to have an anti-tumor effect through their ability to specifically target and then kill cancer cells. [33,34] From this it would logically follow that individuals with increased levels of neutrophils and platelets and/or decreased levels of lymphocytes are at a higher risk of developing cancer.

The results of the present analyses indicate that individuals from the general population who have higher levels of the SII at baseline are more likely to be diagnosed with a solid cancer during follow-up. We showed an increased risk for each subsequent quartile. When exploring the association between the SII and risk of cancer over time, it appeared that the risk increased within the first 6 months of follow-up. This effect could reflect a systemic immune response to a cancer that is already present, however yet undetected. Whether the SII could function as a biomarker for early detection, should be further explored. Studies exploring the effect of changes in the SII over time would be especially insightful. Although we would be cautious in using this marker as a screening tool, since it is a general inflammatory marker and is therefore non-specific.

Despite the fact that the risk is increased in the first 6 months of follow-up, the overall effect cannot merely be explained by reverse causality. The risk persisted after exclusion of data individuals with a follow-up of 6 months or less, and increased when we subsequently evaluated the risk for individuals with a follow-up period of more than 2, 5 or even 8 years of follow-up. This phenomenon supports the hypothesis that chronic inflammation is a risk factor for cancer development. Interestingly, both the innate and adaptive immune system seem to be involved. In which the innate immune system seems to be activated, whereas the adaptive immune system seems to be downregulated. However, whether the inflammatory cells contained in the SII play a causal role in the initiation or the further development of solid tumors, remains to be elucidated. Furthermore, chronic inflammation can be induced by environmental factors. Both smoking and a high BMI are associated with this type of inflammation. Yet we found no effect modification by either of these factors. [3]

To see whether the found effect could be attributed to any specific cancer, we performed a secondary analysis in which alternately the five major solid tumors (colorectal, prostate, breast lung, and bladder cancer) in this population were taken as an endpoint. The effect was present for colorectal, bladder and lung cancer, but was only statistically significant for prostate cancer. We found no effect for breast cancer which may have been due to lack of power, or to differences in tumor biology.

Strengths and Limitations

We showed a relationship between the SII and the diagnosis of a solid cancer in a prospective, population-based cohort, with a long term follow-up of a large number of people. This setting is the design of choice for assessing a relationship between blood levels and the risk of cancer. The association remained robust after adjustment for potential confounders, of which we collected detailed information, and was substantiated by the significant dose-effect relationship as well as an increase of the risk over time.

Ideally, we should have related the SII to the different disease stages. We would hypothesize that individuals with a higher level at baseline were more likely to be diagnosed with metastasized disease and those with relatively lower levels with local disease. [27] Unfortunately, information on stage at diagnosis was not available.

Another limitation was that we had only a single measurement. Multiple measurements over a longer time-period would allow for a more precise measurement and a better understanding of the association. One would be able to better assess whether the SII increases in time up to the diagnosis and could also be used as a marker for early detection.

Lastly, the design of this study did not allow for the assessment of a potential prognostic potential of the SII, although from literature it is known the SII also has prognostic value. [12,13] Recently some studies have also shown that related inflammatory markers, such as the neutrophil-to-lymphocyte ratio may have a predictive value. [28,35] In the future markers such as the SII could help guide therapeutic choices in patients, especially in immunotherapy. [36,37]

In conclusion, the SII is an independent risk indicator for a future diagnosis of a solid cancer on the shorter and longer term. Future studies should further explore and validate this association.

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Supplementary Figure 1. Diagram of examination cycles of the Rotterdam Study. [17]

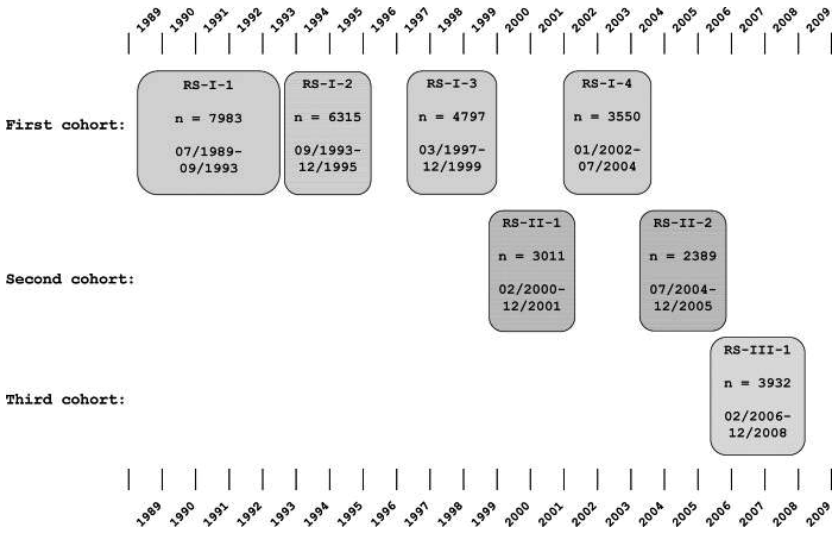
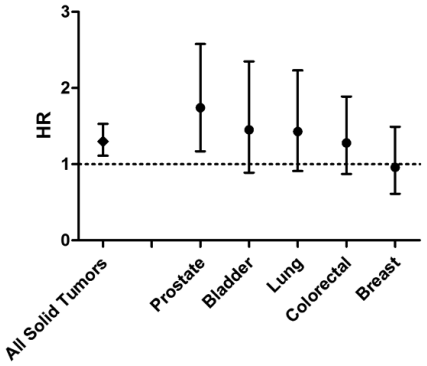


Figure from: The Rotterdam Study: 2010 objectives and design update. Diagram of examination cycles of the Rotterdam Study (*RS*). RS-I-1 refers to the baseline examination of the original cohort (pilot phase 07/1989-12/1989; cohort recruitment 01/1990-09/1993). RS-I-2, RS-I-3 and RS-I-4 refer to re-examination of the original cohort members. RS-II-1 refers to the extension of the cohort with persons in the study district that became 55 years since the start of the study or those of 55 years or over that migrated to the study district. RS-II-2 refers to the re-examination of the extension cohort. RS-III-1 refers to the baseline examination of all persons aged 45 years and over living in the study district that had not been examined (i.e., mainly comprising those aged 45–55 years).

Supplementary Figure 2. Multivariable Cox Proportional Hazard regression for the association between baseline levels of the SII with development of one of the five major solid tumors in this population.



Adjusted for: sub-cohort, sex, age (years), socio-economic status (high/middle/low), smoking status (current/former/never), BMI (body mass index, kg/m^2) and Type II diabetes status. All solid tumors: HR 1.30 (95% CI: 1.11 – 1.53); prostate cancer (men only): HR 1.74 (95% CI: 1.17 – 2.58), bladder cancer: HR 1.45 (0.89 – 2.35) lung cancer: HR 1.43 (0.91 – 2.23), colorectal cancer: HR 1.28 (0.87 – 1.49) and breast cancer (women only): 0.96 (95% CI: 0.61 – 1.49).

Chapter 7

Underestimation of pancreatic cancer in the national cancer registry – reconsidering incidence and survival rates.

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Abstract

Background: In the Netherlands, like in many other European countries, pancreatic cancer mortality was found to be systematically higher than the incidence. This suggests that there is an underestimation of the reported incidence of pancreatic cancer.
Aim: We aimed to study the incidence of pancreatic cancer in the Rotterdam area and to compare this to the national level.

Methods: This study is embedded in the Rotterdam Study, an ongoing population-based prospective cohort study of people aged 45 years and over, enrolled between 1989 till 2006. Details on incident pancreatic cancer cases were available until 2013. Age specific incidence rates were calculated and compared to data available in the Netherlands Cancer Registry.

Results: At baseline 14,922 participants were at risk of developing pancreatic cancer. Median follow-up time was 16.4 person years per person. In total 113 participants developed pancreatic cancer. Rates increased with age with an incidence rate of 109.9 (95% CI; 85.7-138.8) per 100,000 person years for people older than 75. This is higher than the currently reported 55.9 – 89.2 per 100,000 person year. Of the 113 cases identified in the Rotterdam Study, only 67.3% were reported as pancreatic cancer in the Netherlands Cancer Registry. Cases that were not registered, were significantly older and had a significantly poorer survival.

Conclusion: The incidence of pancreatic cancer, as registered by the Netherlands Cancer Registry, is an underestimation. Patients, not registered by the cancer registry, have a significantly poorer survival. Consequently, we probably overestimate the already poor survival of pancreatic cancer.

Introduction

Pancreatic cancer is currently one of the most lethal types of cancer in Europe and has a 5-year survival of around 5%. [1] Due to aging of populations, the incidence of pancreatic cancer has increased over the past few decades in Europe. [1, 2] In the past decade some improvement in survival has been reported, but still [3] it is expected to become the second deadliest cancer by the end of 2020. [2,4]
In line with this European trend, the incidence rate of pancreatic cancer has increased in the Netherlands as well. The estimated incidence rate varies from 0.5-3.6 per 100,000 person years for persons younger than 50 years to 55.9 – 89.2 per 100,000 person years for persons older than 75 years. [5]

In the Netherlands, cancer incidence is registered nationwide by the Netherlands Comprehensive Cancer Registration (IKNL). Cause of death, however, is registered by a different body: Statistics Netherlands. They collect death certificates from the Municipal Personal Records Database (BRP), with date and cause of death as assigned by treating physicians.

Between 2010 and 2014, the number of new cases diagnosed ranged from 2,198 to 2,326. [6] Interestingly, in those same years, fewer patients were diagnosed with pancreatic cancer than died of this cancer (2,481-2,682). [7] In fact, the rate of pancreatic cancer mortality has been systematically higher than the incidence rate, since the start of the Netherlands Cancer Registration (NKR) in 1989. [6]Above numbers suggest an underestimation of the true incidence of pancreatic cancer or an overestimation of pancreatic cancer mortality, which could be important for several reasons. Firstly, because these numbers are supposed to inform clinicians and their patients. Secondly because incidence and mortality rates largely influence the way we prioritize our focus in studying different diseases and lastly, because these numbers are used to advise health care and insurance company policy makers.

The objectives of this study were to establish the incidence rate of pancreatic cancer and its mortality in a large and longstanding population-based prospective cohort study, and to extrapolate this number to a national level to get insight into this discrepancy in figures from national registries.

Patients and Methods

Study Population

The study was embedded in the Rotterdam Study, an ongoing population-based prospective cohort study in the Netherlands. The rationale and design have been described extensively previously. [8,9] Briefly, in 1989 inhabitants of the suburb Ommoord, aged 55 years and older, were invited to participate. The original cohort was enrolled between 1989 and 1993.

Of 10,275 invited subjects, 7,983 entered the study (78%). A second cohort of 3,011 persons (67% response), was enrolled between 2000 and 2001. In 2006 a third cohort with 3,932 persons of 45 years and older were enrolled (65% response). This resulted in an overall study population of 14,926 individuals, aged 45 years and older.

The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports.

Assessment of Cancer Cases

Rotterdam Study

In this study, cases of pancreatic cancer were identified through follow-up of medical records of the general practitioners, by hospital discharge letters, and furthermore through linkage with the Dutch Hospital Data (Landelijke Basisregistratie Ziekenhuiszorg, previously Landelijke Medische Registratie) and registries of histo- and cytopathology. Cases were classified according to the International Statistical Classification of Diseases, 10th revision (ICD-10) and the International Classification of Primary Care, 2nd edition (ICPC-2). [10, 11]

All potential cases of pancreatic cancer and level of certainty thereof, were independently adjudicated by two physicians (JF, RR). In case of disagreement, consensus was sought through consultation of an experienced pancreatic surgeon (CvE).

Level of certainty of diagnosis was established as: certain (pathology confirmed), probable (clinical diagnosis based on a mass in the pancreas and/or liver metastases on CT-scan, ultrasound or endoscopic ultrasonography and/or increased levels of CA19.9) or possible (e.g. an uncircumscribed mass by physical examination or a clinical presentation with painless jaundice and weight loss).

Date of death was obtained through the mortality registry of the Municipal Personal Records Database (Basisregistratie Personen, previously Gemeentelijke Basisadministatie) and cause of death was obtained through follow-up of records of general practitioners or hospital discharge letters. Cause of mortality was coded similarly as morbidity, independently by two physicians according to the ICD-10 and the ICPC-2. [10, 11] All potential cases of pancreatic cancer were provided to the Netherlands Cancer Registry for matching.

Netherlands Cancer Registry

The Netherlands Cancer Registry started registering cancer incidence in 1989. Newly diagnosed malignancies are notified to the Netherlands Cancer Registry by the automated pathological archive (PALGA), supplemented with data from the Dutch Hospital Data. Unlike many other cancer registries, the Netherlands Cancer Registry has no access to notification by death certificates. Information on vital status is regularly obtained from the Municipal Personal Records Database by using a data linkage procedure.

Trained registrars verify all notifications and routinely collect data on patient characteristics, tumor type and primary treatment from medical records in all Dutch hospitals. Tumor location and histology are registered according to the International Classification of Diseases for Oncology (ICD-O-3). [12]

Covariables

The following covariables were considered as potential confounding factors: age, sex, socioeconomic status (high/middle/low), smoking status (current/former/never), alcohol use (heavy [3 or more glasses a day]), moderately [more than once a week, but less than 3 glasses a day], and minor [less than one glass a week]), body mass index (BMI; kg/m^2) and incident diabetes mellitus (fasting glucose ≥ 7.0 mmol/L or use of glucose-lowering medication). [13] Patient characteristics were determined at baseline by interview or during visits at the examination centre.

Statistical Analysis

For each participant, follow-up started at the day of inclusion in the study, until date of cancer diagnosis, death or end of study period (1st of January 2013), whichever came first. To assess differences between cases and the remaining cohort and subsequently between registered and unregistered cases, we used Mann-Whitney tests for continuous variables and χ^2 tests for categorical data.

Incidence and mortality rates with 95% confidence intervals were calculated, both overall and per age category, as described by Rothman et al. [14] Differences in survival between cases from the Rotterdam Study and the Netherlands Cancer Registry, were assessed by Kaplan Meier curves and tested with a Log Rank test and a Wilcoxon test. Significance of associations was accepted at a P-value < 0.05. All analyses were performed using SPSS software (Version 21.0).

Results

General Characteristics Cohort

We used data from all participants of the Rotterdam Study, with the exception of 4 participants who had a history of pancreatic cancer at baseline. At the start of the study 14,922 participants were at risk of developing pancreatic cancer, of whom 6,101 men (40.9%) and 8,821 women (59.1%), with a mean age of 66.0 years (SD 10.5) at baseline.

Table 1. General characteristics for cases stratified by registration in the Netherlands Cancer Registry.

		Total				**Registered**				**Unregistered**				***P*-value**
		N	%	Mean	SD	N	%	Mean	SD	N	%	Mean	SD	
Total		113				82				31				
Sex	Male	44	38.9			34	41.5			10	32.3			0.371
	Female	69	61.1			48	58.5			21	67.7			
Age at Baseline				68.7	8.4			67.4	8.4			72.1	7.4	0.004
Age at Diagnosis				77.3	8.8			75.8	9.1			81.0	6.8	0.005
Follow-up in years	(median, SE)			9.2	0.7			9.2	0.9			9.1	1.1	0.666
Survival in days	(median, SE)			71.0	12.0			90.0	15.0			47.0	13.0	0.009
Socioeconomic Status*	High	13	11.8			10	12.3			3	10.0			0.344
	Middle	38	34.2			24	29.6			14	46.7			
	Low	60	54.1			47	58.0			13	43.3			
Smoking #	Current	32	29.4			27	33.8			5	17.2			0.076
	Former	43	39.4			33	41.3			10	34.5			
	Never	34	31.2			20	25.0			14	48.3			

Table 1. Continued

Alcohol ^	Heavy	16	19.0			13	30.3			3	15.8			0.110
	Moderately	33	39.3			26	49.4			7	36.8			
	Sometimes	35	41.7			27	40.9			8	44.4			
BMI **				26.9	3.8			26.8	3.8			27.1	3.9	0.821
DM-II		26	23.0			18	22.0			8	25.8			0.664
Certainty of diagnosis	Certain	50	44.2			45	54.9			5	16.1			<0.001
	Probable	59	52.2			36	43.9			23	74.2			
	Possible	4	3.5			1	1.2			3	9.7			

* SES: unknown; registered 1, unregistered 1.
\# Smoking: unknown; registered 2, unregistered2.
^ Alcohol: unknown; registered 16, unregistered 13.
** BMI: unknown; registered 7, unregistered 6 .

Total follow-up time was 160,071 person years, with a median follow-up time of 16.4 person years (SE 0.2 person years) per person. Completeness of follow-up until 1st of January 2013 was 98.5%.

Risk of pancreatic cancer

In total 113 participants developed pancreatic cancer: 38.9% male, 61.1% female. Almost all cases were diagnosed above the age of 65 (92.0%), with a mean age at diagnosis of 77.3 years (SD 8.8). In only 44.2% of the cases, diagnosis was confirmed through pathology. Further baseline characteristics can be found in **Table 1**.

At the end of the study period, all patients with pancreatic cancer had died; 94.7% attributable to pancreatic cancer. The median survival was 71.0 days (SE 11.6), with 1-year survival of 11.4% and no patient survived more than 5 years. Most patients (86.8%) did not receive any form of treatment for their pancreatic cancer. Out of the 18 patients undergoing surgery, only 8 had a complete resection of the cancer (7.1%).

The incidence rate and mortality rate for pancreatic cancer in this study population, aged 45 years and older, were calculated at 70.6 and 66.8 per 100,000 person years, respectively. Incidence and mortality rates were also calculated per age category separately (**Table 2 and 3**), the rates increased with age.

Analyses of matching

Of the 113 cases provided to the Netherlands Cancer Registry for matching, 76 cases were registered as cases of pancreatic cancer (67.3%). Seventeen other cases were also registered, however with an unknown primary tumor (n=6, 5.3%) or for a different cancer (n=11, 9.7%). For the latter, most cases were confirmed as patients with double or multiple tumors in the Rotterdam Study. These cancers were non-melanoma skin cancers (n=4), prostate (n=2), breast (n=1), lung (n=1) or colon cancer (n=1). The remaining twenty cases were unknown to the Netherlands Cancer Registry (17.7%).

Patients who were not registered by the Netherlands Cancer Registry were significantly older at time of cancer diagnosis (Mann-Whitney: $p = 0.005$) and were significantly less likely to have had their diagnosis confirmed by pathology (χ^2: $p < 0.001$). Cases from the Rotterdam Study had a significantly poorer overall survival than the cases in the general population as registered by the Netherlands Cancer Registry (Log-Rank: 0.013; Wilcoxon: $p = 0.017$), **Figure 1**. Within the Rotterdam Study, cases that were not registered by the Netherlands Cancer Registry had a significantly lower cancer specific survival than those that were registered (Log-Rank: $p = 0.018$; Wilcoxon: $p = 0.009$), **Supplementary Figure 1**.

Table 2. Incidence rates of pancreatic cancer per age category

Age category	Cases	Percent of cases	Follow-up (Person Years)	Incidence Rate (per 100,000 Person Years)	95% Confidence Intervals (Poisson)
45-54	1	0.9	5,767	17.3	0.4-96.6
55-64	8	7.1	35,060	22.8	9.9-45.0
65-74	34	30.1	55,537	61.2	42.4-85.5
75-84	49	43.4	45,305	108.2	80.0-143.0
≥85	21	18.6	18,405	114.1	70.6-174.4
Overall	113	100	160,074	70.6	58.2-84.9

Table 3. Mortality rates of pancreatic cancer per age category

Age category	PDAC specific mortality	Follow-up (Person Years)	Mortality Rate (per 100,000 Person Years)	95% Confidence Intervals (Poisson)
45-54	1	5,769	17.3	0.4-96.6
55-64	8	35,067	22.8	9.8-45.0
65-74	30	55,549	54.0	36.4-77.1
75-84	47	45,327	103.7	76.2-137.9
≥85	21	18,409	114.1	70.6-174.4
Overall	107	160,121	66.8	54.8-80.8

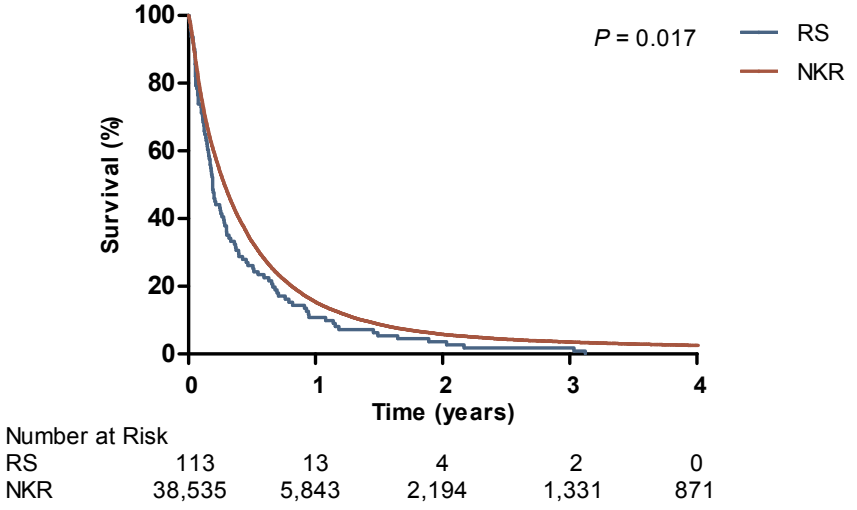

Figure 1. Overall survival curves from cases in the Rotterdam Study (RS) versus cases in the general population as registered by the Netherlands Cancer Registration (NKR)

Discussion

In the Rotterdam Study, of the approximately 15,000 individuals aged 45 years and older who were followed during 160,071 person years, 113 patients developed pancreatic cancer. We calculated the overall incidence rate at 70.6 per 100,000 person years for this specific population and showed that the rate increased with age. They parallel the age specific incidence rates as reported by Coupland et al., but are higher. [15] We expect that in the Netherlands, around 3,000-3,750 people develop pancreatic cancer annually. This is far more than the approximately 2,500 that are currently registered. [6]

We showed that of the 113 patients who developed pancreatic cancer, only 67.3% was registered by the Netherlands Cancer Registry as pancreatic cancer. This confirms our assumption that there is an underestimation of the incidence rate as registered by the Netherlands Cancer Registry. This does not only hold true for the Netherlands. In multiple other European countries, amongst which Belgium, Iceland and Sweden, the reported incidence rate is lower than the mortality rate of pancreatic cancer. [16]

Another part of the discrepancy between the national incidence and mortality, might be explained by misclassification of cause of mortality. Compared to the European mean, the mortality of pancreatic cancer is higher in the Netherlands, while the mortality of cholangiocarcinoma is lower. [6] However this is no explanation for the grove under registration we showed in this study.

Most cases that were not registered by the cancer registry, did not have pathological confirmation of the cancer, suggesting that the cancer registry relies heavily on pathological verification. [17] This might be particularly problematic for pancreatic cancer. Pathological confirmation for all cases in this cohort was 44.2%, compared to 54.9% when only analyzing cases registered by the Netherlands Cancer Registry. This last number is more in line with earlier reported verification rates of 57.0% and 62.7%. [18, 19]. Histopathological confirmation rates have significantly risen over the past years, so the relatively low rate in this study can be partly explained by the long follow-up period of this study. [19] Even though pancreatic cancer has one of the lowest verification rates of all cancers, our data suggest that potential inflation of these percentages occurred. [17-19] Patients who had had their diagnosis confirmed by pathology were significantly younger (data not shown). It is plausible that in elderly patients, in the light of a poor prognosis or a poor clinical condition, prohibiting any palliative treatment, patients and their treating physicians consider additional invasive diagnostics too burdensome.

Indeed, we showed that pancreatic cancer is a disease of the elderly, with the highest incidence and mortality figures in the age category of 85 years and above.

Lastly, we showed that cases that were not registered had a significantly poorer survival than those that were. This means we do not only underestimate the incidence of pancreatic cancer, but also overestimate the survival. The overall survival in this cohort is dramatic: the 1-year survival was only 11.4% and no patient survived more than 5 years. In 94.7%

death was attributable to pancreatic cancer. This can partly be explained by the stage of disease at presentation, as stage of disease heavily influences survival. [20]. Only 7.1% was able to undergo successful surgery. All surgeries were performed after 2000. Most patients were treated by oncologists from one local hospital and were unlikely to be referred to a tertiary center, once the disease was locally advanced or metastasized, to undergo any form of palliative treatment. Almost 25% of the patients died within a month after diagnosis and were unlikely to be candidates for palliative chemotherapy such as Gemcitabine or FOLFIRINOX (folinic acid, fluoracil, irinotecan, oxaliplatin). Furthermore, FOLFIRINOX was only introduced at the very end of the study period and, although it might improve survival, it is unlikely that it had much impact on survival in this study. [21, 22]

Strengths and limitations

Strengths of this study are the prospective design, the duration and completeness of follow-up and , most importantly, the completeness of the registration of cancer cases. What sets apart cancer registration in the Rotterdam Study, is the additional information that is obtained from follow-up of medical records of general practitioners. For pancreatic cancer, there is a considerable group of patients for whom diagnosis is not pathologically confirmed or who are not admitted to hospital. These patients are therefore missed by the currently available notification sources of the Netherlands Cancer Registry. Completeness of the Netherlands Cancer Registry could be enhanced by information on cause of death, as collected by Statistics Netherlands. However if patients die from another cause, either truly or as documented, while diagnosed with pancreatic cancer, chances are that these patients will still be missed by the Netherlands Cancer Registry. Therefore investment in the gathering of more detailed information on cancer morbidity is probably most effective in ensuring better coverage.

The Rotterdam Study consists of individuals of 45 years and older, the age groups in which pancreatic cancers occurs most frequently. However, as a consequence we were not able to calculate an age standardized incidence rate. Another limitation is that a long follow-up period automatically means that part of the data is old, therefore not always reflecting the effects of new insights, diagnostics and therapies. This holds for pathological verification of disease, but also for treatment of pancreatic cancer with palliative chemotherapy. However, our data were compared to national data from the same time period. The observed differences therefore cannot be explained by these limitations.

In conclusion, the incidence of pancreatic cancer, as registered by the Netherlands Cancer Registry, is an underestimation. Patients that are not registered by the cancer registry are significantly less likely to have had their diagnosis confirmed by pathology, are significantly older and have a poorer survival. Consequently, besides underestimation of the incidence, we are also likely to overestimate the already poor survival of pancreatic cancer.

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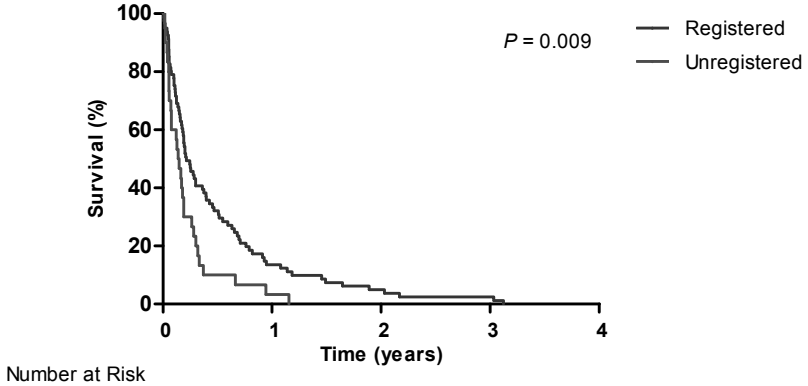
Supplementary Figure 1. Kaplan Meier Curves for pancreatic cancer cases in the Rotterdam Study, stratified for registration by the Netherlands Cancer Registry



Number at Risk					
Registered	82	12	4	2	0
Unregistered	31	1	0	0	0

Chapter 8

The systemic immune-inflammation index as a marker for the impairment of the immune system in pancreatic cancer prior to diagnosis.

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In preparation

Abstract

Background: The systemic immune-inflammation index (SII) is an inflammatory marker that reflects the inflammatory status. It is a prognostic factor in patients with resectable pancreatic cancer. Pancreatic cancer is capable of misleading the immune system in such a way that it enables the cancer to progress. We used the SII to assess if there were changes in the immune system in the time prior to detection of the cancer.

Methods: Data were obtained from a population-based prospective cohort study of individuals aged ≥ 45 years. Absolute blood counts were used to calculate the SII: P*N/L. We used prospectively collected measurements from the Rotterdam Study in addition to measurements collected from hospital files. We used a mixed linear model to assess whether the SII measurements changed over time. Analyses were adjusted for age, sex, smoking, socio-economic status, diabetes and body mass index.

Results: During a median follow-up time of 16.9 years, 122 out of 14,922 participants developed pancreatic cancer. Mean age at diagnosis was 77.7 years (SD 8.9). At diagnosis of pancreatic cancer, SII levels were higher; with a median of 1440 (IQR: 726–3033) compared to the median SII in the overall population (455; IQR: 339– 618). For 49 cases diagnosed with pancreatic cancer, we had SII measurements in the two years prior to diagnosis. There was a significant increase of the SII during that time (*P*-value = 0.0183), also after adjustment for potential confounders (*P*-value = 0.0085).

Conclusion: These results show that the immune system is affected by the presence of pancreatic cancer even prior to diagnosis. Further research should investigate whether the SII could be used for the early detection of pancreatic cancer or therapeutic response.

Introduction

Pancreatic cancer is predicted to be the second deadliest cancer by 2030. (1) Only 15-20% of the patients can be operated with curative intent. However, even in these patients and despite intensive adjuvant treatment, there is recurrence of disease, leaving the 5 year overall survival at around 4-7%. (2, 3) Recent developments in chemotherapeutic treatments have certainly helped improving survival, however only for a limited group of patients who are fit enough to undergo these regimens. (4) Immunotherapy has proved to be a successful treatment in aggressive cancers such as melanoma and lung cancer and might also be of benefit in pancreatic cancer. (5, 6) Therefore, recently more effort has been put into understanding the role of the immune system in pancreatic cancer.

It is well recognized that the immune system plays an important role in cancer surveillance and the elimination of tumor cells. (7-9) However, it is also known that pancreatic cancer is capable of misleading the immune system in such a way that it no longer attacks tumor cells, but rather forms a supportive structure for the cancer. (10, 11) Furthermore, pancreatic cancer has a local immunosuppressive environment that is ideal for tumor growth. (12)

To gain insight into the biology of pancreatic cancer and for the development of potential immunotherapeutic agents, examination of the role of inflammatory markers in these tumors has become more pressing. One of these markers is the systemic immune-inflammation index (SII). (13, 14) The SII is a relatively newly recognized inflammatory marker, comprising the peripheral neutrophil, lymphocyte and platelet counts. (13) A high SII results from either an increase in neutrophils and platelets or a decrease in lymphocyte counts, and reflects an imbalance of the host inflammatory status. It has been established to be a strong prognostic factor for survival in patients with hepatocellular carcinoma as well as in patients with resectable pancreatic cancer. (13, 14) Furthermore we recently demonstrated that healthy individuals with relatively high levels of the SII have an increased risk to develop a solid cancer. The risk of diagnosis with a solid cancer was highest in the first 6 months after blood measurement, suggesting that there is an impairment of the immune system already prior to the detection of cancer. (15) Therefore, the aim of this study was to investigate whether SII levels change prior to the diagnosis of pancreatic cancer.

Methods

Study Setting

The study was embedded in the Rotterdam Study, an ongoing population-based prospective cohort study in the Netherlands. The rationale and design have been described extensively previously. (16, 17) Briefly, in 1989 inhabitants of the suburb Ommoord, aged 55 years and older, were invited to participate. The original cohort was enrolled between 1989 and 1993.

Of 10,275 invited subjects, 7983 entered the study (78%). A second cohort of 3011 persons (67% response) was enrolled between 2000 and 2001. In 2006, a third cohort with 3932 persons of 45 years and older were enrolled (65% response). This resulted in an overall study population of 14,926
individuals aged 45 years and above.

Participants were visited at home at baseline for a standardized interview on health status. Subsequently, a physical examination followed with extensive laboratory and imaging procedures during a visit at the study center. These interviews and visits were repeated approximately every four years.

Assessment of pancreatic cancer cases

Cases of pancreatic cancer were identified through follow-up of medical records of the general practitioners, by hospital discharge letters and furthermore through linkage with the Dutch Hospital Data and registries of histo- and cytopathology. Additionally, cases were verified in the national cancer registry. Cases were classified according to the International Statistical Classification of Diseases, 10th revision (ICD-10) and the International Classification of Primary Care, 2nd edition (ICPC-2). (18, 19)

All potential cases of pancreatic cancer were independently assessed by two physicians [JF and RR]. In case of disagreement, consensus was sought through consultation of an experienced pancreatic surgeon (CvE).

Date of death was obtained through the mortality registry of the municipality and the cause of death was obtained through follow-up of records of general practitioners or hospital discharge letters. The cause of mortality was coded similarly as morbidity, independently by two physicians according to the ICD-10 and the ICPC-2. (18, 19)

Assessment of the SII

In the Rotterdam Study, fasting blood samples were collected at the study center and full blood count measurements were performed immediately after blood draw. These measurements included absolute counts of granulocytes, lymphocytes, and platelets and were performed using the COULTER® Ac·T diff2™ Hematology Analyzer (Beckman Coulter, San Diego, California, USA). However the leukocyte differential measurements were only part of the protocol from the fourth visit of the first cohort onwards (see **Supplementary Figure 1**). (20) Additionally, since study center visits occurred roughly every four years the number of SII measurements from the Rotterdam Study was limited. Therefore, we subsequently collected absolute counts of granulocytes, lymphocytes and platelets from patient files in the hospitals where they had been treated. If the blood measurements contained percentages for the leukocyte differential, we calculated the absolute number of granulocytes and lymphocytes from the absolute leukocyte count.

The systemic immune-inflammation index (SII) was calculated from the platelet (P; $x10^9$/Liter), granulocyte, as a proxy for neutrophils, (N; $x10^9$/Liter), and lymphocyte (L; $x10^9$/Liter) blood counts, using the following formula: SII = P x N/L. (13)

Assessment other covariables

The following variables were considered as potential confounding factors: age, sex, socio-economic status (high/intermediate/low), smoking status (current/former/never), and body mass index (BMI; kg/m^2). Individual characteristics were determined at baseline by interview or at the study center and roughly every four years at every consequent follow-up visit.

Status on prevalent type 2 diabetes was ascertained from general practitioners' records (including laboratory glucose measurements), hospital discharge letters, and serum glucose measurements at the study center. Diabetes was defined, according to the WHO guidelines, as a fasting glucose ≥ 7.0 mmol/Liter or use of glucose – lowering medication.

Statistics

Patient characteristics were presented as numbers and percentages for categorical variables and means with standard deviations for continuous variables. The SII was not normally distributed, therefore we presented the median with interquartile ranges (IQR).

We calculated the time between the dates of SII measurements and date of diagnosis in years. We ran a linear regression model to see if the time between SII level measurements and diagnosis corresponded with SII levels in the measurements performed in the Rotterdam Study.

To assess the change of the SII over time we included the measurements collected from the patient files and used a linear mixed model to assess the change of the SII in the time prior to diagnosis. This model allows for the examination of repeated measurements with varying time intervals within one person. Unlike a logistic regression or a Cox proportional hazard model, this model does not provide an effect estimate, but rather indicates whether the change of the SII over time (slope) is statistically significant or not. We therefore reported *P*-values only.

We ran a sensitivity analysis to explore the change in the SII in the two years prior to diagnosis of pancreatic cancer. This analysis was additionally corrected for smoking, SES, diabetes and BMI. As these parameters are also subject to change over time, we did not use the baseline measurements of these variables, but the measurements that concurred or occurred closest to the time of blood sampling. Prior to any of the analyses the SII was log-transformed, because it had a non-normal distribution.

Significance of associations was accepted at a *P*-value < 0.05. Analyses were performed using SPSS software (Version 21.0) and R Version 3.1.3.

Results

General characteristics pancreatic cancer patients

At the start of follow-up, 14,922 participants were at risk to develop pancreatic cancer. All were followed until the 1st of January 2015. During a median follow-up time of 16.9 years, 122 participants were diagnosed with pancreatic cancer. The majority was female (60.1%) and the mean age at diagnosis was 77.7 years (SD 8.9). Further baseline characteristics can be found in **Table 1.**

Table 1. Patient characteristics at baseline.

Characteristic		**N (%)**	**Mean (SD)**
Total		122 (100)	
Sex	Male	48 (39.3)	
	Female	74 (60.7)	
Age at baseline (years)			68.5 (8.4)
Age at diagnosis (years)			77.7 (8.9)
SES	High	14 (11.5)	
	Intermediate	37 (30.3)	
	Low	69 (56.6)	
Smoking	Current	36 (28.7)	
	Former	47 (38.5)	
	Never	36 (29.5)	
BMI (kg/m^2)			27.0 (3.8)
Diabetes	Yes	13 (10.7)	
	No	109 (89.3)	

Percentages do not add up to 100% due to missing values.

Most patients presented with advanced disease. Nineteen participants underwent a surgical exploration, however in five patients the cancer had metastasised and in another five the disease was locally too advanced, so only nine patients were operated and underwent a resection with curative intent (7.4%). Seven participants underwent palliative chemotherapy and one chemo-radiation therapy. The majority (84.4%) did not receive any form of therapy.

All patients died during the follow-up of the study. No patient lived longer than 5 years and the median survival was 69 days (SE 11 days), see **Figure 1**.

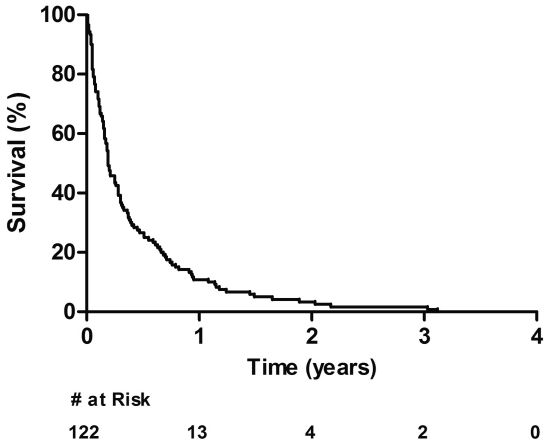

Figure 1. Kaplan – Meier curve for the survival of pancreatic cancer patients.

SII measurements over time

We had SII measurements for 8,024 out of 14,922 participants, including 75 of the 122 that developed pancreatic cancer. For the pancreatic cancer patients this resulted in a total of 188 measurements. Of these 64 were collected during the regular follow-up of the Rotterdam Study and the other 124 were collected retrospectively from the patient files of the treating hospitals. Of the latter 34 measurements were taken more than one month after diagnosis and were therefore excluded from the analyses, leaving a total of 154 SII measurements that were used in the main analysis (see **Figure 2 and Supplementary Table 1**).

We found that the SII at diagnosis with a median of 1440 (IQR; 526 – 3033), was almost three times higher when compared to SII levels in the 5 – 10 years prior to diagnosis of pancreatic cancer (Median 523; IQR: 417 – 635) or to the general population (Median 455; IQR: 339 – 618). **Table 2**, **Figure 2**. These results are much influenced by measurements from the patient files (see **Supplementary Table 1**).

In the Rotterdam Study measurements only, the time till diagnosis did not correspond with SII levels, both in the crude linear model and the age and sex adjusted model (*P*-value = 0.978 and 0.806, respectively). Next, we included measurements that were collected from the patient files and used a linear mixed model to see whether the SII significantly increased over time until the diagnosis (*P*-values < 0.001 for both the crude and sex and age adjusted model).

In the two years prior to diagnosis levels raised above the upper reference limit of the SII in the general population (see **Figure 2**). Therefore, we ran a sensitivity analysis for 49 patients of whom we had measurements in the two years prior to diagnosis (n = 89, measurements from the Rotterdam Study and the patient files combined). The SII also significantly increased during this time period in the fully adjusted model (*P*-value$_{\text{fully adjusted model}}$ = 0.009).

Table 2. SII measurements over time all measurements.

Time prior to diagnosis of pancreatic cancer	Number of Patients	Number of Measurements	SII Median (IQR)
In the general population	8,024	8,024	455 (339 – 618)
At diagnosis only	28	40	1440 (526 – 3033)
<6 months	33	51	1165 (535 – 2448)
6 months – 2 years	25	38	594 (434 – 1312)
2 – 5 years	25	34	597 (376 – 727)
5 – 10 years	27	29	523 (417 – 635)
> 10 years	2	2	-

SII = systemic immune-inflammation index, IQR = inter quartile range.

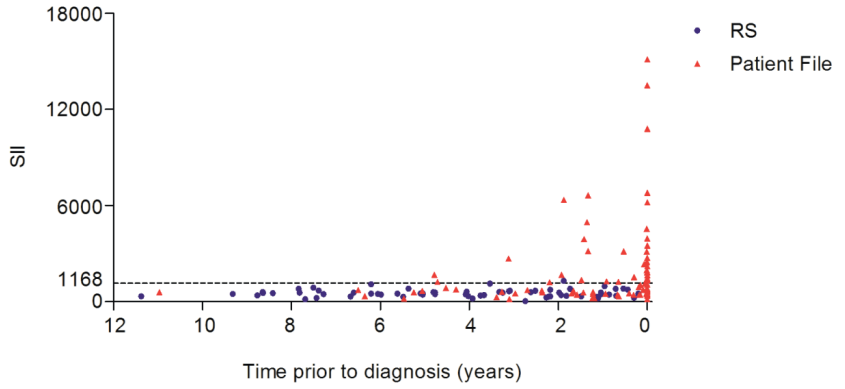

Figure 2. SII measurements over time prior to the diagnosis of pancreatic cancer
For 75 of the 122 pancreatic cancer patients we had at least 1 blood measurement resulting in a total of 154 measurements. Of these 64 were collected during the regular follow-up of the Rotterdam Study (blue) and the other 124 were collected retrospectively from the patient files of the treating hospitals (red).
N.B. Five measurements are outside of the Y-axis (outliers).
The upper reference limit has been previously established in this population (20).

Discussion

It is well known that inflammation plays an important role in the development of cancer. A healthy immune system acts as a surveillance system for recognizing and eliminating circulating tumor cells. (7-9) However, it is also known that in patients with pancreatic cancer the immune system no longer functions properly. Cancer cells can evade immune-surveillance and it is even thought that pancreatic cancer can modulate the immune system in such a way that it forms a supportive structure for the cancer cells. (10, 11, 21)

However, little is known on how and when the immune system changes over time in people who are later diagnosed with pancreatic cancer. Therefore, we used the SII as a biomarker of a potential impairment of the innate and the adaptive immunity and assessed SII levels in pancreatic cancer patients both prior to, and at the time of diagnosis. We found that SII levels were higher in patients with pancreatic cancer than in the general population, whereas initially they had comparable SII levels to the general population. The increase in SII levels mainly occurred in the two years prior to diagnosis. The high SII resulted from an increase in neutrophils and platelets and a decrease in lymphocyte counts, suggesting an impairment of the immune system.

In general, inflammation of malignant tumors is associated with a poorer survival. (22) Pancreatic cancer, in particular, is associated with nonspecific inflammation that is generally not effective against the cancer itself. (10, 11, 21) There are also changes in the immune system that seem to allow the cancer to thrive. For instance, lymphopenia is common in pancreatic cancer and a decrease in $CD8^+$ lymphocytes is the result of an increase of FoxP3+ T regulatory cells. They produce several immune inhibiting molecules which create a local immunosuppressive environment that allows for tumor growth. Additionally, the low levels of lymphocytes systemically allow for the dissemination of more tumor cells through the circulation. (10, 12, 23)

In contrast, both neutrophils and platelets are thought to have a promoting effect on cancer. Platelets may adhere to cancer cells protecting them from cell death when traveling in the circulation and platelet-derived factors help cells to migrate from the blood stream into visceral organs. (24, 25)

Neutrophils were traditionally considered innocent bystanders in the cancer setting. (26) More recently it has been assumed, however, that neutrophils may be important in tumor initiation, progression, and metastasis. (26, 27) In pancreatic cancer specifically, a recent study showed that in mice with pancreatic cancer the primary tumor produces TIMP-1 (tissue inhibitor of metalloproteases), which caused neutrophils to be recruited to the liver, where they stimulated the liver to from pre-metastatic niches. (28, 29) These mice had a faster progression of disease and had a poorer survival.

The added value of the SII in clinical practice remains unclear however there is increasing evidence that the immune status of patients with cancer could predict response to either chemo- or immunotherapy. (30) In patients with suspected pancreatic cancer it is sometimes

difficult to differentiate between pancreatic cancer and benign disease such as chronic pancreatitis or auto-immune pancreatitis. Further research should investigate whether the SII could be used in the diagnostic work-up of patients with suspected pancreatic cancer without definitive histopathological diagnosis.

Strengths and limitations

This is the first study to assess the SII of patients with pancreatic cancer prior to diagnosis. Moreover, we have collected multiple measurements which allows for the assessment of the change in the SII over time, giving us insight into when the impairment of the immune system can be measured. Lastly, patients were selected from a prospective population-based cohort study which limited the chance of selection or information bias. Therefore, this study also includes patients with locally advanced and metastasized disease and is not just limited to patients with potentially resectable disease.

However, there are some limitations that warrant mentioning. Firstly, part of the data was collected retrospectively and the results of this study were mostly driven by these retrospectively collected data. Consequently, we only have information on patients that were admitted to hospital. Therefore, our findings might not be generalizable to patients with pancreatic cancer who were not admitted to hospital. Secondly, we only have blood samples available. Although this provides us with insight into the systemic immune status of patients, this may not reflect the tumor immune interaction in the its direct tumor environment. Ideally, we would have liked to correlate these findings to tumor tissue which was not available. Lastly it would be insightful to compare the level of SII to circulating tumor load, but unfortunately these data were not available.

In conclusion, these results show that the SII may be a useful marker for the presence of pancreatic cancer even prior to diagnosis. Further research should investigate whether the SII could be used for the early detection of pancreatic cancer or for therapeutic response.

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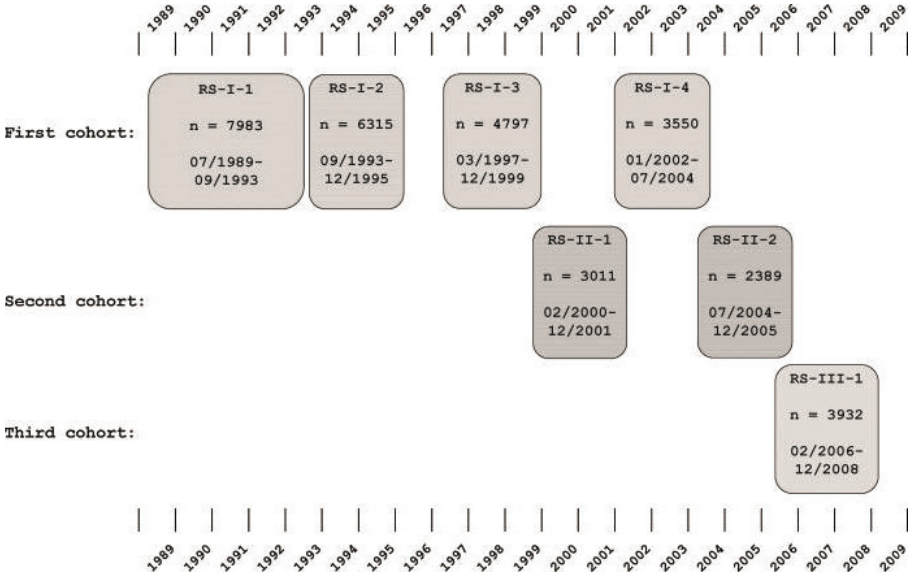
Supplementary Table 1. SII measurements over time separated for Rotterdam Study and patient files.

Time prior to diagnosis of pancreatic cancer	Number of Measurements Rotterdam Study	SII Median (IQR) Rotterdam Study	Number of Measurements Patient Files	SII Median (IQR) Patient Files
At diagnosis only	-	-	40	1440 (726 – 3033)
<6 months	3	516 (376 – 612)	48	1350 (625 – 2502)
6 months – 2 years	15	472 (362 – 806)	23	675 (497 – 3164)
2 – 5 years	21	569 (345 – 635)	13	553 (720 – 1219)
5 – 10 years	24	517 (438 – 598)	5	581 (278 – 694)
> 10 years	1	-	1	-

SII = systemic immune-inflammation index, IQR = inter quartile range.

Supplementary Figure 1. Study center visits in the Rotterdam Study.



Chapter 9

Search for Early Pancreatic Cancer Blood Biomarkers in Five European Prospective Population Biobanks Using Metabolomics.

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Endocrinology, 2019

Abstract

Background: Most patients with pancreatic cancer present with advanced disease and die within the first year after diagnosis. Predictive biomarkers that signal the presence of pancreatic cancer in an early stage are desperately needed. We aimed to identify new and validate previously found plasma metabolomic biomarkers associated with early stages of pancreatic cancer.

Methods: Prediagnostic blood samples from individuals who were to receive a diagnosis of pancreatic cancer between 1 month and 17 years after sampling (N = 356) and age- and sex-matched controls (N = 887) were collected from five large population cohorts (HUNT2, HUNT3, FINRISK, Estonian Biobank, Rotterdam Study). We applied proton nuclear magnetic resonance–based metabolomics on the Nightingale platform.

Results: Logistic regression identified two interesting hits: glutamine (P = 0.011) and histidine (P = 0.012), with Westfall–Young family-wise error rate adjusted P values of 0.43 for both. Stratification in quintiles showed a 1.5-fold elevated risk for the lowest 20% of glutamine and a 2.2-fold increased risk for the lowest 20% of histidine. Stratification by time to diagnosis suggested glutamine to be involved in an earlier process (2 to 5 years before diagnosis), and histidine in a process closer to the actual onset (<2 years).

Conclusion: Our data did not support the branched-chain amino acids identified earlier in several US cohorts as potential biomarkers for pancreatic cancer. Thus, although we identified glutamine and histidine as potential biomarkers of biological interest, our results imply that a study at this scale does not yield metabolomic biomarkers with sufficient predictive value to be clinically useful per se as prognostic biomarkers.

Introduction

Pancreatic cancer is one of the most lethal cancers worldwide and is increasingly common (1–3). Most patients present with advanced and thus incurable disease and die within a year of the initial diagnosis (3, 4). There is an imminent need to identify these patients earlier in the disease process, as patients with resectable, nonmetastatic cancer can potentially be cured. For many cancers it takes several years for a local malignant lesion to progress to fully metastasized disease, and pancreatic cancer is no exception (5). Thus, there should be a window of opportunity for timely detection and intervention. Unfortunately, for early, presymptomatic pancreatic cancer currently no specific biomarkers are available. The identification of predictive biomarkers is complicated by the low incidence rate of the disease, estimated at 7 to 12 cases per 100,000 adult person years in the Western European population (6, 7).

It is well known that the development and progression of pancreatic cancer are associated with alterations in systemic metabolism. Patients may present with glucose intolerance, anorexia, and severe weight loss (3, 8). In line with this, circulating metabolites have been proposed as a potentially useful screening tool in pancreatic cancer (9–16). The study by Mayers et al. (11) stood out from other metabolomic biomarker studies, as they analyzed blood samples taken 2 to more >10 years prior to diagnosis. They found an elevation of circulating branched-chain amino acids as an early event in the development of pancreatic cancer (11).

Considering these metabolomics biomarkers as promising, we set out to replicate these findings independently in five large European population cohorts and find additional biomarkers associated with early stages of pancreatic cancer, using a different platform, proton nuclear magnetic resonance (1H-NMR) instead of liquid chromatography followed by mass spectrometry. This is a retrospective study where biobanked samples from population cohorts were cross-checked with the national cancer registries to identify samples from individuals who were diagnosed with pancreatic cancer after blood sampling. This was done because truly prospective studies are almost infeasible with low-incident diseases such as pancreatic cancer.

Methods

Study population

Our study population consisted of pancreatic cancer cases and controls, drawn from five national European cohorts, collaborating in the Biobanking and Biomolecular Resources Research Infrastructure Large Population Cohorts (BBMRI-LPC; www.bbmri-lpc-biobanks.eu) and the cross-infrastructure project CORBEL (www.corbel-project.eu): the Estonian Genome Center of the University of Tartu study (EGCUT), the FINRISK Study (FR), the Nord-Trøndelag Health Study (HUNT2 and HUNT3), and the Rotterdam Study (RS).

EGCUT is a volunteer-based sample of the Estonian resident adult population aged ≥18 years, started in 1999, and currently has nearly 152,000 participants (17). EGCUT can link its own database with the national electronic databases (eight total) to constantly update the phenotype information for the subjects. Every entry in the biobank consists of: (i) biological samples, (ii) answers to the questions of a computer-assisted personal interview conducted at the doctor's office, (iii) objective measurements performed at the doctor's office, (iv) electronic health data from various databases, (v) genotype data from array genotyping, exome sequencing, or whole-genome sequencing, and (vi) biomedical data obtained by performing various assays on the material collected.

FINRISK was initiated in 1972 and includes a collection of cross-sectional surveys in the adult (25- to 74-year-old) permanent residents of selected geographical areas of Finland. Altogether, FINRISK had nine cross-sectional surveys performed every fifth year by the National Institute for Health and Welfare, including a total of 101,451 invitees (18). Participants in this study were selected from the FINRISK 1997, 2002, and 2007 surveys. There are no reexaminations except for occasional people who were selected to more than one independent survey by chance. Follow-up is carried out through record linkages to national administrative registers (such as the Causes of Death Register and Cancer Register) by using a unique personal identity code (19).

HUNT includes repeated surveys of a large population-based cohort in Norway. Data from 116,044 individuals aged ≥20 years from HUNT2 (1995 to 1997, n = 65,237) and HUNT3 (2006 to 2008, n = 50,807) were used in this study. Individuals who participated in both HUNT2 and HUNT3 were only included in the current study as part of HUNT3. Similar to FINRISK, follow-up is carried out through record linkages to national administrative registers (such as the Causes of Death Register and Cancer Register) by using a unique personal identity code (20).

The RS is an ongoing, population-based cohort study in a suburban area of Rotterdam, Netherlands. At baseline, all participants underwent both an interview at home and an extensive set of examinations at a research facility, and blood samples (both plasma and serum) were collected. At each follow-up point, blood samples were collected. It was initiated in 1989 and has enrolled 14,926 individuals of ≥45 years of age since then. Follow-up is carried out every three to four years. An automated follow-up system is linked to digital medical records from general practitioners (including discharge letters from hospitals) and linked to a registry of histopathology and cytopathology [Pathologisch-Anatomisch Landelijk Geautomatiseerd Archief (PALGA)] and to Landelijke Medische Registratie (LMR) and the Integraal Kankercentrum Nederland (IKNL) (21, 22).

All participants of the respective cohorts provided written informed consent. The current study was approved by the local ethics committee of each study.

Selection of cases and controls

We included incident pancreatic cancer cases, confirmed by pathology and diagnosed after blood collection. Cases were identified through national cancer registries and through independent review of medical records. For diagnosis of pancreatic cancer, we used the ICD-10 C25.0 code. Deaths were ascertained through the national registries. We excluded cases that lived >5 years after diagnosis to avoid false-positive diagnoses (23–25).

For each case, we selected two (in RS one, in EGCUT four) random controls, matching on cohort, sex, age at sample collection (±2 years), and time of blood collection. Controls were those who were alive and without a diagnosis of pancreatic cancer at time of the case's diagnosis date.

Ascertainment of other covariates

The following covariate data were obtained from questionnaires and physical examination before blood collection: body mass index (BMI; kg/m2), smoking status (current/former/never), type 2 diabetes mellitus (T2DM) status, and fasting status (<4 hours/4 to 8 hours/>8 hours).

Metabolite profiling and quality control

Serum was collected from serum separator tubes with glass or silica clot activators, with or without gel as separator, and stored at −80°C. EDTA plasma was collected from Vacutainer tubes and processed and stored at −80°C within 48 hours of blood draw. Metabolites were quantified from EDTA plasma (EGCUT) or serum (HUNT2, HUNT3, FR, RS) samples using a high-throughput 1H-NMR metabolomics platform (Nightingale Health, Helsinki, Finland; https://nightingalehealth.com/). This platform provides simultaneous quantification of 147 individual metabolites and 79 metabolite ratios, for example, routine lipids, lipoprotein subclass profiling with lipid concentrations within 14 subclasses, esterified fatty acid composition, and various low-molecular-weight metabolites, including amino acids, ketone bodies, and gluconeogenesis-related metabolites in molar concentration units. Details of the experimentation and applications of the platform have been described previously (26).

Metabolite measures that failed quality control (in particular for glutamine, pyruvate, glycerol, hydroxybutyrate, and acetate) were excluded from the analysis on a per-individual basis. One metabolite measure (glycerol) with >10% missing values was excluded entirely, resulting in a final number of 146 metabolite measures and 79 ratios. Outliers (>5 SD) were removed in concordance with previous research in this field (27).

Statistical analysis

Differences in baseline characteristics between cases and controls were assessed for each cohort separately using two-tailed Student t tests (continuous variables) or $\chi 2$ tests (categorical variables).

Metabolite measurements were raised by 1 to allow log transformation. Thereafter, all metabolite values were log-transformed and scaled to obtain unit SD for each cohort. They were included as continuous variables in logistic regression models and adjusted for matching factors (sex and age at sample collection, minimally adjusted model). In our main model on the pooled data from all of the cohorts, we additionally adjusted for BMI, smoking status, T2DM status, fasting status, and cohort. P values were corrected for multiple testing using Westfall and Young family-wise error rate, an appropriate method given the strong correlations between the measurements of the different metabolites (28). To provide estimates of effect magnitude, significant metabolites were again examined in logistic regression models after categorization in quintiles. Quintiles were generated based on the metabolite values in controls only. Results are presented as ORs and 95% CIs.

As an alternative for the pooling of the data from the different cohorts, we also performed a logistic regression per cohort (with sex, age, BMI, smoking status, T2DM, and fasting status as covariates) and a subsequent meta-analysis. The obtained estimates for the metabolite measures and their standard errors were used in a random effects meta-analysis using the R package meta 4.9.2 (29). A random effects model was chosen to account for possible heterogeneity due to differences in disease assessment, sample processing, and sample collection between cohorts. Heterogeneity was assessed using the I2 statistic and by visual inspection of forest plots. P values from the meta-analysis were corrected for multiple-testing using a Bonferroni–Holm test.

LASSO regression to evaluate additive effect of metabolomics biomarkers on top of clinical predictors

To select biomarkers with predictive value, we applied a fivefold cross-validated penalized least absolute shrinkage and selection operator (LASSO) regression with the penalized package version 0.9-51 (30). The clinical covariates (sex, age, BMI, smoking status, T2DM status, fasting status, and cohort) were not penalized and thus were always present in the model. We performed a stratified analysis, including all controls but only cases who developed pancreatic cancer within 2 years or within 5 years after blood sampling or including all cases. For the variable selection, the data were split randomly into a data set for variable selection (70% of the data, with 35% for training and 35% for cross-validation) and a data set for performance testing (30% of the data). We compared the performance of the null model (with only the clinical covariates) with the model that included the

selected metabolites using an ordinary least squares regression model. The performance of the different model was assessed by evaluating the area under the receiver operator curve (AUC).

General

Analyses were performed using the software packages meta 4.9-2, Penalized 0.9-51, Globaltest 5.24.0, InformationValue 1.2.3, ROCR 1.0-7, RColorBrewer 1.1-2, and ggplot2 3.0.0 for R version 3.2.3. All scripts are available in an online repository (31).

Availability of data

Additional files with complete results are available in an online repository (32). For reasons of privacy protection, raw data are only available upon request.

Results

Study population and measurements

Cross-checking of the individuals in the five population cohorts included in this study with the national cancer registries enabled us to identify 444 prediagnostic samples from subjects who received diagnosis of pancreatic cancer between 1 month and 17 years after blood sampling (median, 4.68 years). We subsequently selected 1012 sex- and age-matched controls from the same cohorts (Fig. 1). Baseline characteristics for all cohorts are shown in Table 1. Baseline characteristics differed significantly between cohorts (in particular for sex, BMI, T2DM, and fasting status). Cases were significantly and consistently enriched for T2DM patients and smokers, in line with the comorbidity of pancreatic cancer and T2DM and smoking as a known risk factors for pancreatic cancer (Table 1). We reliably quantified 146 blood metabolites and 79 metabolite ratios. Figure 1 shows the number of participants remaining after quality control and after assessment of the completeness of phenotype information in the different analyses performed.

Single-metabolite logistic regression

To identify metabolite biomarkers potentially associated with future pancreatic cancer diagnosis, we performed a separate logistic regression for each metabolite measured. In our primary model, we adjusted for the following covariates: sex, age, BMI, smoking status, T2DM status, fasting status, and cohort. The results of our top metabolites are presented in Table 2. Full data are provided in an online repository (32). Two metabolites demonstrated

Table 1. Baseline Characteristics

	HUNT2 (n=590)			HUNT3 (n=194)			EGCUT (n=227)			FR (n=272)			RS (n=173)			ALL (n=1456)		
	Cases	Controls	Statistics	Cases	Controls	Statistics	Cases	Controls	Statistics	Cases	Controls	Statistics	Cases	Controls	Statistics	Cases	Controls	Statistics
Total, n (%)	158 (26.8%)	432 (73.2%)		64 (33%)	130 (67%)		76 (33.5%)	151 (66.5%)		57 (21%)	215 (79%)		89 (51.4%)	84 (48.6%)		444 (30.5%)	1012 (69.5%)	
Female, n (%)	80 (50.6%)	220 (50.9%)	p = 0.95	37 (57.8%)	76 (58.5%)	p = 0.93	44 (57.9%)	88 (58.3%)	p = 0.96	22 (38.6%)	82 (38.1%)	p = 0.95	54 (60.7%)	45 (53.6%)	p = 0.35	237 (53.4%)	511 (50.5%)	p = 0.31
Age (years), mean (SD)	66.8 ± 11.1	64.8 ± 11.2	p = 0.065	69.5 ± 9.5	69.5 ± 9.3	p = 0.97	63.4 ± 10.6	63.3 ± 10.5	p = 0.94	59.7 ± 8.8	59.8 ± 8.9	p = 0.96	71.6 ± 8.5	70.9 ± 9.2	p = 0.62	66.6 ± 10.7	64.6 ± 10.8	p = 0.001*
BMI (kg/m^2), mean (SD)	27.3 ± 4.1	26.9 ± 3.7	p = 0.28	28.3 ± 3.6	27 ± 4.2	p = 0.025*	29.1 ± 5.8	28.5 ± 5.4	p = 0.40	27.2 ± 3.9	27.7 ± 4.3	p = 0.45	27.4 ± 4.1	27.3 ± 4.1	p = 0.89	27.8 ± 4.4	27.3 ± 4.3	p = 0.078
DM, n (%)	11 (7%)	18 (4.2%)	p = 0.16	10 (15.6%)	8 (6.2%)	p = 0.034*	33 (43.4%)	32 (21.2%)	p = 0.00059*	6 (10.5%)	21 (9.8%)	p = 0.86	12 (13.5%)	2 (2.4%)	p = 0.0074*	72 (16.2%)	81 (8%)	p = 2.77e-06*
Smoking, n (%)	0 = 49 (31%) 1 = 48 (30.4%) 2 = 59 (37.3%) NA = 2 (1.3%)	0 = 187 (43.3%) 1 = 140 (32.4%) 2 = 93 (21.5%) NA = 12 (2.8%)	p = 0.00041*	0 = 22 (34.4%) 1 = 19 (29.7%) 2 = 20 (31.3%) NA = 3 (4.7%)	0 = 58 (44.6%) 1 = 55 (42.3%) 2 = 13 (10%) NA = 4 (3.1%)	p = 0.00077*	0 = 38 (50%) 1 = 18 (23.7%) 2 = 20 (26.3%) NA = 0 (0%)	0 = 100 (66.2%) 1 = 20 (13.2%) 2 = 31 (20.5%) NA = 0 (0%)	p = 0.044*	0 = 21 (36.8%) 1 = 14 (24.6%) 2 = 19 (33.3%) NA = 3 (5.3%)	0 = 106 (49.3%) 1 = 66 (30.7%) 2 = 41 (19.1%) NA = 2 (0.9%)	p = 0.043*	0 = 22 (24.7%) 1 = 37 (41.6%) 2 = 27 (30.3%) NA = 3 (3.4%)	0 = 20 (23.8%) 1 = 50 (59.5%) 2 = 12 (14.3%) NA = 2 (2.4%)	p = 0.021*	0 = 152 (34.2%) 1 = 136 (30.6%) 2 = 145 (32.7%) NA = 11 (2.5%)	0 = 471 (46.5%) 1 = 331 (32.7%) 2 = 190 (18.8%) NA = 20 (2%)	p = 8.12e-09*
Fasted, n (%)	0 = 134 (84.9%)	0 = 363 (84%)		0 = 46 (71.9%)	0 = 97 (74.6%)		0 = 35 (46.1%)	0 = 97 (64.2%)		0 = 4 (7%)	0 = 4 (1.9%)		0 = 26 (29.2%)	0 = 21 (25%)		0 = 245 (55.2%)	0 = 582 (57.5%)	
0=<4h, 1= 4h-8h, 2= >8h	1 = 19 (12%) 2 = 3 (1.9%) NA = 2 (1.3%)	1 = 56 (13%) 2 = 9 (2.1%) NA = 4 (0.9%)	p = 0.95	1 = 9 (14.1%) 2 = 6 (9.4%) NA= 3 (4.7%)	1 = 18 (13.8%) 2 = 5 (3.8%) NA = 10 (7.7%)	p = 0.32	1 = 7 (9.2%) 2 = 6 (7.9%) NA = 28 (36.8%)	1 = 24 (15.9%) 2 = 15 (9.9%) NA = 15 (9.9%)	p = 0.87	1 = 38 (66.7%) 2 = 14 (24.6%) NA = 1 (1.8%)	1 = 171 (79.5%) 2 = 40 (18.6%) NA = 0 (0%)	p = 0.052	1 = 1 (1.1%) 2 = 56 (62.9%) NA = 6 (6.7%)	1 = 0 (0%) 2 = 57 (67.8%) NA = 6 (7.1%)	p = 0.50	1 = 74 (16.7%) 2 = 85 (19.1%) NA = 40 (9%)	1 = 269 (26.6%) 2 = 126 (12.5%) NA = 35 (3.5%)	p = 1.49e-05*

Values are number counts (percentages) or mean ± standard deviation. BMI = Body mass index, DM = Diabetes mellitus.

* Chi-square test

** T-test

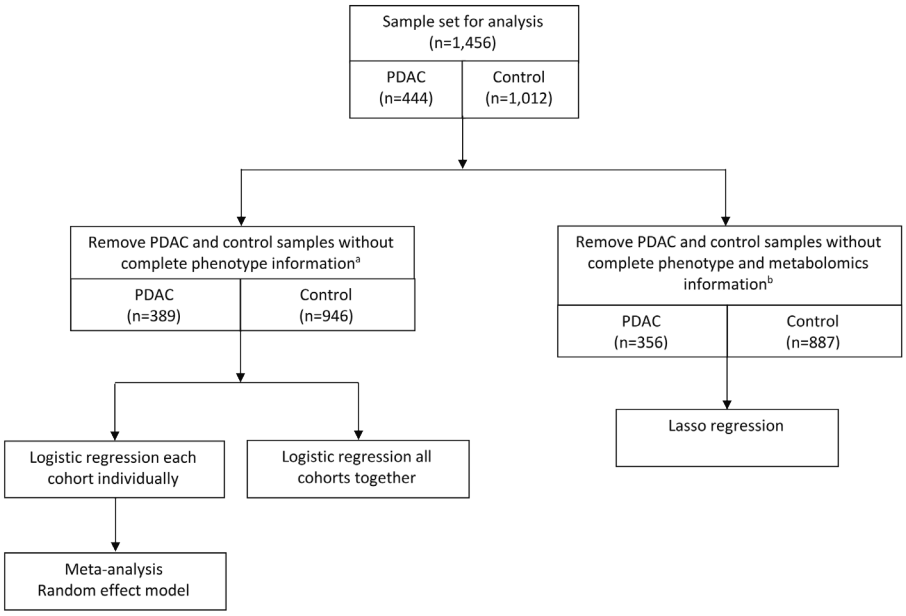

Figure 1. Flowchart PPC
Determination of the sample set used for data-analysis and the different data-analysis approaches performed in the current study.
a Any individual containing missing values in metabolomics measurements or phenotypically information were assumed to be missing at random, and removed from the dataset.
b Any individual containing missing values in phenotypically information were removed from the dataset.

lower blood levels in cases than in controls and nominal significance: glutamine (P = 0.012) and histidine (P = 0.011). They were not significant after adjustment for multiple testing (Westfall–Young family-wise error rate adjusted P value of 0.43 for both metabolites). To estimate the clinical relevance of our findings, the ORs for being diagnosed with pancreatic cancer within the follow-up time was calculated for an individual with metabolite levels of 1 SD below the mean: these ORs amounted to 1.42 and 1.45 for glutamine and histidine, respectively (see footnote to Table 2). A closer inspection of the levels of glutamine and histidine revealed that the differences were consistently observed across cohorts (Fig. 2A and 2E), except for glutamine in RS and histidine in FR. Glutamine levels were lower in both nondiabetics and patients with diabetes, whereas lower histidine levels were mainly observed in patients with pancreatic cancer who were also diagnosed with T2DM (Fig. 2B). Histidine levels were lower in individuals who developed pancreatic cancer within 2 years after blood sampling, whereas glutamine levels were decreased longer before diagnosis (Fig. 2C and 2G). Histidine levels were lower in both fasting and nonfasting individuals (Fig. 2H), whereas the effect of fasting status on glutamine levels is difficult to ascertain given the differences between cohorts in fasting

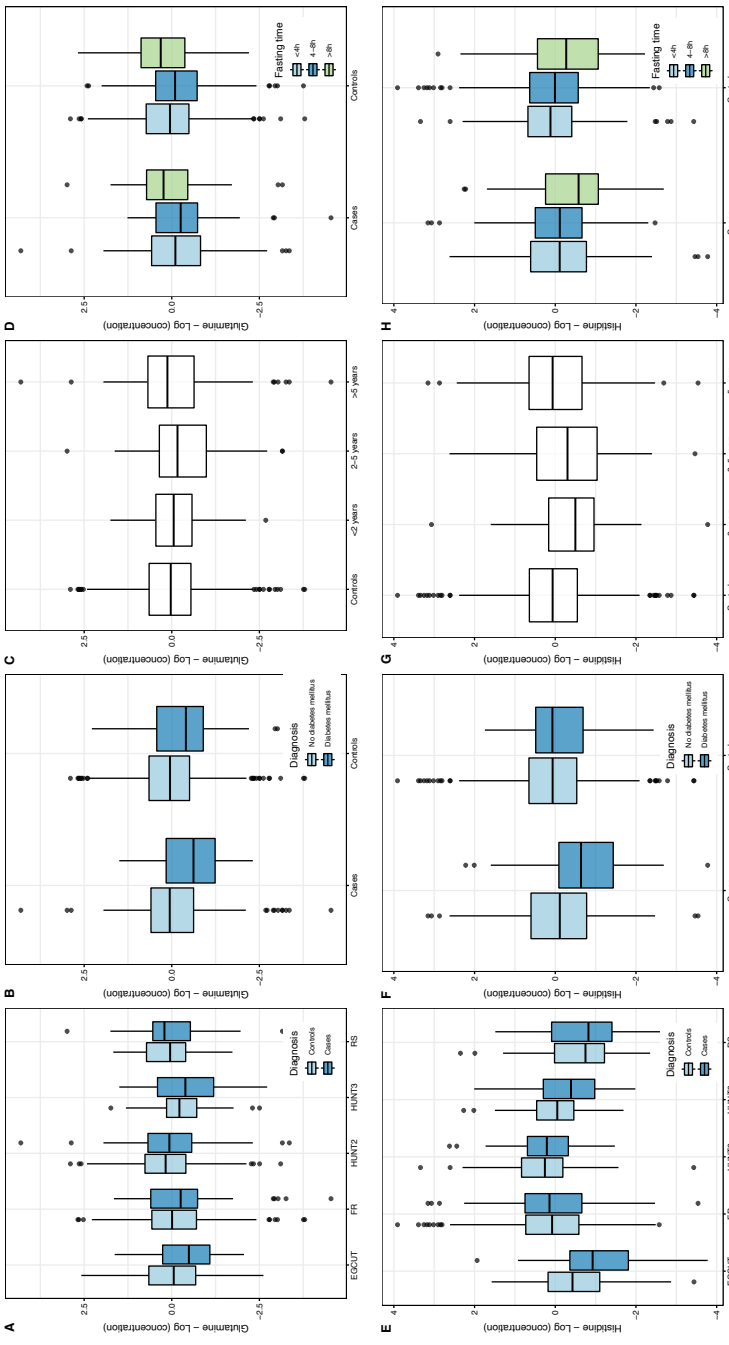

Figure 2.
Concentrations (logarithmic scale) of (A–D) glutamine and (E–H) histidine in the blood circulation in controls and cases, that is, those individuals who developed pancreatic cancer within a time window after blood sampling. (A and E) Distribution of the concentrations of controls (light blue) and cases (dark blue) in the different cohorts analyzed (EGCUT, FR, HUNT2, HUNT3, RS). (B and F) Distribution of concentrations in nondiabetics (light blue) and individuals diagnosed with T2DM (dark blue). (C and G) Distribution of concentrations in controls and cases sampled within 2 y before diagnosis, between 2 and 5 y before diagnosis, and >5 y before diagnosis. (D and H) Distribution of concentrations in nonfasting individuals (light blue), individuals who had a meal between 4 and 8 h before blood draw (dark blue), and fasting individuals (green, last meal was >8 h before blood draw). Box plots reflect the distribution of the concentrations in individual samples, including the middle quartiles (25th to 75th percentile of the data points are in the boxes); the horizontal band; the median value; the lower whiskers representing the data points up to 1.5 × the interquartile range (IQR) below the 25th percentile; the upper whiskers representing the data points up to 1.5 × IQR above the 75th percentile; the data points outside these ranges plotted as individual data points.

status (Fig. 2D). The branched chain amino acids, leucine, valine, and isoleucine, reported earlier by Mayers et al. (11), were not different between cases and controls (unadjusted P values of 0.75, 0.94, and 0.61, respectively).

The results above were recapitulated in a minimally adjusted model, only corrected for sex and age (32). Glutamine and histidine were still among the top hits, with P values of 0.0063 and 0.00045 (not adjusted for multiple testing), respectively.

To further address potential cohort differences, we performed a meta-analysis on the β coefficients from the logistic regression models that were applied per cohort. The results are summarized in Table 3 and provided in full in an online repository (32). The results from the meta-analysis corroborated our findings on the pooled data, with lower glutamine levels seen for all cohorts (unadjusted P value of 0.0040), but most prominently in HUNT3 (Fig. 3A), and lower histidine levels mostly in HUNT3 and EGCUT (unadjusted P value of 0.0022) (Fig. 3B, with similar trends in other cohorts and evidence for significant heterogeneity between cohorts). The mean ORs for an increase of 1 SD in glutamine or histidine levels were 0.82 and 0.78 (or 1.22 and 1.28 for a decrease of 1 SD), respectively. The meta-analysis provided some evidence for the involvement of ω-3 fatty acids (including docosahexaenoic acid and high-density lipoproteins).

To provide a better understanding of the lower glutamine or histidine levels, we stratified the cohorts in quintiles based on the glutamine or histidine levels in controls. Individuals within the lowest 20% of glutamine levels ran a 1.5-fold elevated risk of pancreatic cancer, and individuals within the lowest 20% of histidine levels ran a 2.2-fold elevated risk of pancreatic cancer (Table 4).

Table 2. Top Hits From Logistic Regression Analysis

Metabolite[a]	Estimate[b]	SE	*z* Value	*P* Value	Adjusted *P* Value
Histidine	−0.188	0.074	−2.529	0.011	0.4274
Glutamine	−0.175	0.069	−2.525	0.012	0.4274
DHA.FA	0.195	0.081	2.393	0.017	0.5214
FAw3.FA	0.17	0.075	2.272	0.023	0.6203
M.HDL.P	−0.151	0.072	−2.085	0.037	0.7646
M.HDL.L	−0.149	0.072	−2.076	0.038	0.7695
DHA	0.153	0.075	2.029	0.043	0.7975
M.HDL.PL	−0.145	0.072	−2.020	0.043	0.8016
M.HDL.C	−0.139	0.071	−1.941	0.052	0.8513
M.HDL.CE	−0.138	0.072	−1.929	0.054	0.8589
M.HDL.PL	0.141	0.074	1.898	0.058	0.8756

Abbreviations: DHA, docosahexaenoic acid; HDL, high-density lipoprotein; DHA.FA, ratio of docosahexaenoic acid to all fatty acids; FAw3.FA, ratio of ω-3 fatty acids to total acids; M.HDL.P, concentration of medium HDL particles; M.HDL.PL: phospholipids in medium-sized HDL particles.

[a]Logistic regression with single metabolite measure, sex, age, BMI, smoking status, T2DM status, fasting status, and cohort as covariates.

[b]The estimates are the fitted β coefficients from the logistic regression model. As the input metabolite data were scaled, the estimates can be interpreted as follows: the OR for developing pancreatic cancer in a case with a typical low metabolite score of 1 SD below the average z score (= −1) would amount to 1.22 for β of −0.1 and 1.49 for β of −0.2. The z value mentioned in the table is the test statistic from the logistic regression models.

Table 3. Top Hits From Meta-Analysis

Metabolite	β	CI	Unadjusted *P* Value	*P* Value	I^2 (%)
Glutamine	−0.19538	−0.33:−0.06	0.004	0.9037087	0
DHA.FA	0.17259	0.04:0.3	0.0083	1	0
M.HDL.PL	−0.17905	−0.32:−0.04	0.0103	1	0
M.HDL.P	−0.17856	−0.32:−0.04	0.0104	1	0
M.HDL.L	−0.17732	−0.31:−0.04	0.0104	1	0
FAw3.FA	0.16222	0.03:0.29	0.0126	1	0
Histidine	−0.25164	−0.46:−0.05	0.0156	1	0.53
M.HDL.FC	−0.15636	−0.29:−0.02	0.0251	1	0
M.HDL.C	−0.15174	−0.29:−0.02	0.0267	1	0
M.HDL.CE	−0.14723	−0.28:−0.01	0.0306	1	0
DHA	0.13222	00:00.3	0.0438	1	0

Abbreviations: DHA, docosahexaenoic acid; DHA.FA, ratio of docosahexaenoic acid to total fatty acids; FAw3.FA, ratio of *ω*-3 fatty acids to total acids; HDL, high-density lipoprotein; M.HDL.C, total cholesterol in medium-sized HDL particles; M.HDL.CE, cholesterol esters in medium-sized HDL particles; M.HDL.FC, free cholesterol in medium-sized HDL particles; M.HDL.L, total lipids in medium-sized HDL particles; M.HDL.P, concentration of medium-sized HDL particles; M.HDL.PL, phospholipids in medium-sized HDL particles.

[a]Meta-analysis across the five cohorts of logistic regression results with single metabolite measure, sex, age BMI, smoking status, T2DM status, and fasting status as covariates. β is effect size and can be interpreted as detailed in footnote *b* to Table 2. *P* value is Bonferroni–Holm-adjusted *P* value. I^2 is the statistic used for heterogeneity between cohorts.

Table 4. ORs for Developing Pancreatic Cancer in Different Glutamine and Histidine Strata

	Based on Control Data	Controls (n)	Cases (n)	OR	5% CI	95% CI	*P* Value
Glutamine							
0%	0.269	180	94	1	—	—	
20%	0.4538	176	66	0.72	0.49	1.05	0.0852
40%	0.487	177	62	0.67	0.46	0.98	0.0404
60%	0.5157	176	71	0.77	0.53	1.12	0.1734
80%	0.55358	178	62	0.66	0.46	0.98	0.0376
Histidine							
0%	0.03927	178	110	1	—	—	
20%	0.060498	177	71	0.65	0.45	0.93	0.0199
40%	0.064778	177	58	0.53	0.36	0.78	0.0011
60%	0.068174	177	66	0.6	0.42	0.87	0.0073
80%	0.072638	178	51	0.46	0.31	0.69	0.0001

Table 5. Variables Selected by the LASSO Regression.

Cohort	Time condition	Lambda	Selected variables	*P*-value	Significance
Full data	2 years	6.06	M.VLDL.FC_., UnSat, SFA.FA	0.175	
Full data	5 years	28.3	S.VLDL.FC_., Gln	0.0114	*
Full data	max(t)	2.02	XL.VLDL.TG, XL.HDL.TG, M.HDL.PL, XXL.VLDL.PL_., XXL.VLDL.CE_., L.VLDL.PL_., L.VLDL.FC_., M.LDL.TG_., XL.HDL.CE_., XL.HDL.FC_., L.HDL.FC_., FreeC, SM, LA, DHA. FA, LA.FA, Glc, Cit, Ala, Gln, His, Val, Phe, AcAce, bOHBut, Crea	0.102	

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1

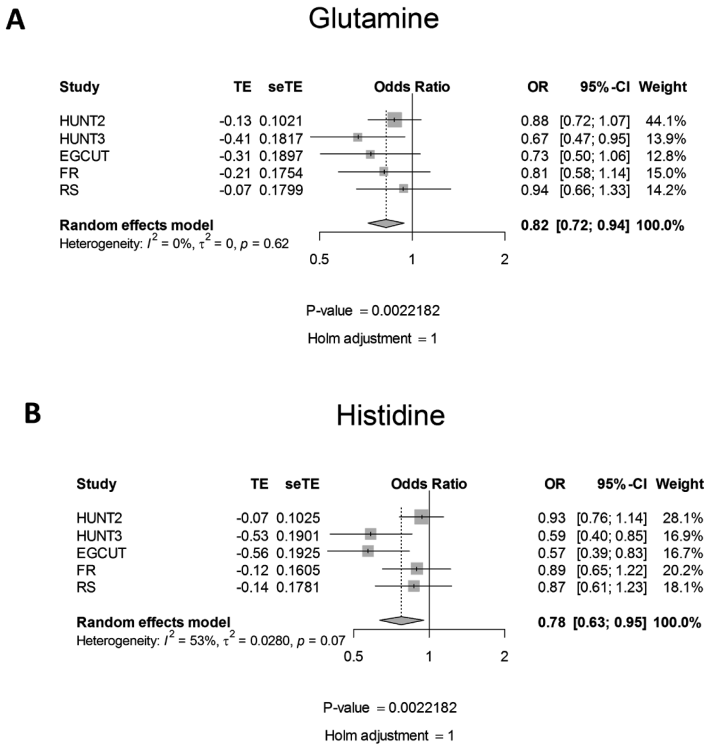

Figure 3.
Forest plots from random effects meta-analysis across different cohorts for (A) glutamine and (B) histidine. The meta-analysis was performed on the β coefficients and SD from the logistic regressions run for each cohort separately. In the logistic regression, pancreatic cancer status was modeled as a function of log-transformed and standardized metabolite concentration, sex, age, BMI, smoking status, T2DM, and fasting status. Shown are the estimated effect size, the SE on this estimate, the estimated OR and the CI on this ratio, the weight of the individual cohort on the calculation of the final estimate, the heterogeneity measure (modeling differences between cohorts), and the unadjusted and Bonferroni–Holm-corrected *P* values for the respective metabolites.

LASSO regression

LASSO regression was used to evaluate the additional predictive value of metabolomics biomarkers over clinical predictors. The performance of a reference (null) model, in which only the clinical covariates were used for prediction, was compared with an alternative model, in which metabolites selected by the LASSO regression were added to the model. The cases were stratified according to the time until diagnosis (up to 2 years, up to 5 years, and all cases without temporal constraint). In the model with cases up to 2 years until diagnosis, the LASSO regression selected medium very low-density lipoprotein (VLDL), total unsaturated fatty acids, and saturated fatty acids to be included in the model (Table 5), but it did not affect the performance on the 30% of the data that were unseen during the selection of the metabolites. In the model with cases up to 5 years until diagnosis, the LASSO regression model selected small VLDL and glutamine (consistent with the prominent decrease of glutamine levels in cases between 2 and 5 years before diagnosis) (Table 5). The performance of the alternative model increased slightly for both the training (AUC of 0.72 vs 0.71 for the null model, Fig. 4A) and the validation set (AUC of 0.64 vs 0.62 for the null model, Fig. 4B). In the model with all cases included, more metabolites were selected (Table 5), but the performance of the model including the metabolites on both training and validation set (AUC of 0.68 and 0.62, respectively) was worse than for the model with cases up to 5 years.

Discussion

Pancreatic cancer is usually diagnosed in an advanced stage of the disease, resulting in a poor prognosis. Most pancreatic cancer biomarker studies executed until today (9, 10, 13–16) collected samples at the time of diagnosis or even later, and therefore have limited clinical utility. However, they may provide insight in the pathophysiology of the disease. The setup of our study allowed for the identification of biomarkers in individuals who were not yet diagnosed with pancreatic cancer, and made efficient use of the large-scale biobanking infrastructure in Europe (BBMRI-LPC program).

We identified two potential biomarkers, glutamine and histidine, while noting that the differences between cases and controls were small and did not survive stringent multiple testing procedures, and that the clinical utility of these biomarkers is currently low. The increased risk of pancreatic cancer associated with low levels of glutamine and histidine was calculated to be only 1.5-fold to 2.2-fold and does not add much in terms of predictive potential to well-known risk factors for pancreatic cancer such as age, smoking, and T2DM. However, also earlier studies provided evidence for alterations in glutamine and histidine in pancreatic cancer (10, 15, 16, 33), suggesting that these may indeed be associated with pancreatic cancer–associated changes in metabolism. In the largest study by Fukutake *et al.* (15) (N = 360 vs 8372), histidine was found particularly low in patients with resectable

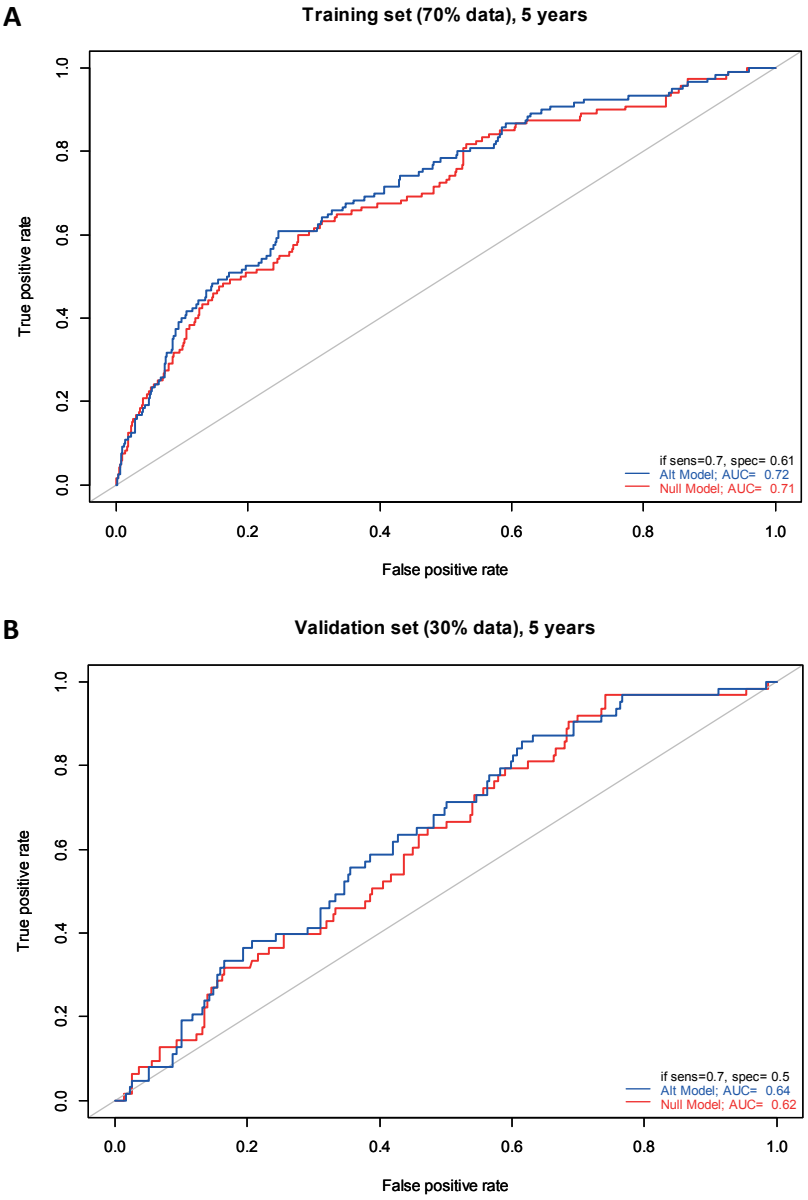

Figure 4.
Receiver operator curves for classification of pancreatic cancer cases (sampled up to 5 y before diagnosis) and controls for (A) training set (70% of all individuals) and (B) performance testing set (30% of all individuals unseen during the variable selection). In red, the null model is shown in which only the clinical covariates (sex, age, BMI, smoking status, T2DM, and fasting status) were included in the regression. In blue, the alternative model is shown where the metabolites selected by the LASSO regression were included in addition to the clinical covariates. The AUCs are indicated, as well as the specificity (1 – false-positive rate) at 70% sensitivity.

disease stage 0-IIB. This group of patients in a relatively early state of the disease is likely most similar to our group of individuals who were diagnosed in <2 years after blood sampling and had the lowest histidine levels of all cases. Also, in other cancer-related studies, negative correlations between histidine levels and cancer incidence and/or cancer-associated mortality were observed (34–36). Remarkably, a recent report demonstrated also lower efficacy of cancer treatment in individuals with low histidine levels, and suggested histidine supplementation to enhance the efficacy of methotrexate treatment in leukemia (37). In a study by Roux *et al.* (33), human pancreatic ductal adenocarcinoma (PDAC) cell lines displayed higher glutamine uptake and metabolism than did non-PDAC cancer cell lines, in line with our study. Moreover, mouse models in which human PDAC cells were injected into the pancreas demonstrated lower levels of circulating glutamine than control animals, which could not be explained by inflammation of the pancreas nor by the development of T2DM (33). This makes it unlikely that the identification of glutamine and histidine in our study is due to pancreatitis, often associated with pancreatic cancer, but we can only formally exclude this possibility by including a cohort of patients with chronic inflammation of the pancreas.

One of the reasons why changes in metabolites such as glutamine and histidine are difficult to detect is that the concentrations of these metabolites are relatively high, and that local events, such as a pancreatic tumor, contribute only little to the overall pool of these metabolites. Other metabolites may be more specific to the metabolism in the pancreas and may show more prominent changes. These types of metabolites require broader metabolomic screening than the Nightingale platform provides. Although having superior robustness and throughput and low cost, the range of metabolites measured on the Nightingale platform is limited to amino acids, other polar metabolites, and a large range of lipid and lipoprotein classes. Our study calls for the use of complementary biomarker platforms on these samples, and suggests to limit the sampling to within 5 years before diagnosis and not beyond.

Our study was not able to replicate the findings from the single study with a design and sample size comparable to ours (11). This study identified the branched-chain amino acids valine, leucine, and isoleucine as potential prognostic biomarkers for pancreatic cancer. We did not find any difference between cases and controls for these amino acids, nor were our top metabolites identified in this earlier study. This may be a reflection of the limited power of both studies for the discovery of small changes observed for these metabolites. However, we did not even observe trends in the same directions. Differences in the measurement platforms (^{1}H-NMR vs liquid chromatography followed by mass spectrometry) may play a role, but the different amino acids can robustly be measured by both. It is equally plausible that the differences are due to differences in the studied populations or confounding factors, which were not or were incompletely corrected for in the statistical model, such as nutrition.

In conclusion, our study lends initial support to the existence of metabolic alterations in early pancreatic cancer development, highlighting glutamine and histidine as metabolites of interest, but also underscores the challenges to find robust, prognostic biomarkers for rare disorders. To address this, larger studies are needed, including more metabolites with lower concentrations and/or integrated studies at multiple "omics" levels.

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Chapter 10

General summary, discussion and future perspectives

General summary

The human immune system is one of the defence mechanisms against invasion of foreign pathogens, but it also has the ability to detect and eliminate damaged- and tumour cells. [1,2] As we age, the immune system alters and becomes less effective at regulating inflammatory processes. This is thought to contribute to an increase in the incidence of morbidity in the elderly population. [1,2]

Additionally, the ageing process seems to be accompanied by a hyper-inflammatory state. This is exemplified by the increased presence of inflammatory markers, such as C-reactive protein (CRP) and IL-6, in the elderly. [3,4] These markers are also associated with increased morbidity and mortality. [5,6] However it is unclear whether these changes of inflammatory mediators are the consequence of the normal ageing process and a declining functioning of the immune system or whether they are caused by pre-existing conditions and thus can be seen as indicators of disease.

The aim of this thesis was to gain a better understanding of the role of the immune system in patients with cancer. In order to do so we studied inflammation-related markers in relation to cancer incidence and mortality both in the healthy, ageing population as well as in patients with cancer.

Probably, the most studied marker of inflammation is CRP. It is considered a surrogate marker of the activation of the immune system. [6] A marker that might provide a reflection of the functioning of the immune system is the leukocyte count. It is known that the total-leukocyte count is associated with an increased morbidity and mortality. [7] However, its subsets: neutrophils and lymphocytes might play different and potentially opposite roles. [7-9] For instance, in the cancer setting, neutrophils may be important in tumour initiation, progression, and metastasis. [10,11] Lymphocytes, on the other hand, are thought to have an anti-tumor effect through their ability to specifically target and then kill cancer cells. [12,13]

The neutrophil-to-lymphocyte ratio (NLR) is a composite marker of subtypes of the total-leukocyte count. [14] It is calculated by dividing the absolute neutrophil count by the absolute lymphocyte count and was developed to study the effect of a subset of white blood cells simultaneously. Like the leukocyte count, it provides a reflection of the functioning of the immune system and allows for the studying of the immune system in an ageing population. [14]

We obtained reference values for the NLR in the Rotterdam Study. Not only were mean NLR levels higher for the higher age groups, over time the NLR increased intra-individually (**chapter 3**). For a long time, it was thought that the innate immune system did not change with age.[15] Although it is now accepted that also the innate immune system undergoes alterations, most studies suggest that there are no changes in neutrophil numbers. [16] However, we found that both neutrophil and lymphocyte counts changed over time and

therefore the NLR increased. Whether this increase is part of the normal ageing process and thus neutrophils increase naturally, or whether it is part of an inflammatory response as a result of disease is still unclear.

In **chapter 4** we showed that individuals with a relatively higher NLR at baseline compared to those with a lower NLR had a higher all-cause mortality. This association was robust after adjustment for clinically relevant confounders and unlikely to explained by any residual confounding.

Although the relationship might be causal, it may also be that the NLR is a proxy indicator of the ageing process or rather a manifestation of an underlying disease. Over time risk of mortality decreased, which may the result of depletion-of-susceptibles. Meaning, healthy individuals live longer, while individuals with an underlying illness or poor health status are censored because they die relatively early in the follow-up. Contradicting this hypothesis, is that even after adjustment for diabetes, history of cancer and history of cardiovascular disease the associations remained. Furthermore, even if there is a depletion-of-the-susceptible over time, the risk continues to exist even for individuals with at least a follow-up period of eight years or more.

Regardless of the nature of the association, the NLR could potentially be used as a marker to stratify patients into risk-groups of those who have a high versus a low mortality risk. However, its clinical value needs to be determined. In this light it is important that future studies take into consideration that the NLR is a non-specific marker of inflammation and the information on NLR levels alone will not provide enough information to estimate the individual risk of mortality. For example, over one third of the individuals in the 99th percentile of the NLR measurements, were still alive after 10 years of follow-up.

A clinically more established marker of inflammation is the erythrocyte sedimentation rate (ESR). It is well known that the ESR can be moderately elevated in the elderly, especially in women. [17,18] It was even thought that moderately elevated levels of inflammation could be disregarded in patients of age. [19] In **chapter 5**, we have shown that the ESR is a robust marker for overall mortality, even when it is only moderately increased and regardless of age. An increased ESR at an older age should therefore not be disregarded but instead warrants further clinical follow-up.

After we studied the role of inflammation in the normal ageing process and mortality, we further investigated the role of inflammation in cancer.

CRP is probably the most extensively studied inflammatory marker in the field of cancer research, despite this its role is still unclear. [20,21] The association between an increased CRP and risk of cancer may have several explanations. Firstly, the possibility that increased CRP levels are a direct cause of cancer. Secondly, that CRP may be a surrogate marker of inflammation where inflammation induces a malignancy. Or, lastly, an increased CRP is the result of a diagnosed cancer or of a cancer that is already present but is yet undetected. [20,21]

To elucidate the role of CRP in cancer, we performed a review and meta-analysis of prospective cohort studies (**chapter 2**). We retrieved high-quality, population-based, prospective studies addressing this issue. Unfortunately, there was a large variety in the methods of analysing the relationship between CRP and cancer. Therefore only a small number of studies could be pooled in the meta-analysis. Nevertheless we found that there is a significant association between CRP and cancer incidence, specifically for lung and breast cancer.

Several studies performed a lagged sub-analysis in which cases during the first few years of follow-up were excluded. [22-25] In these studies the associations either remained or were only slightly attenuated, indicating that the relationship cannot merely be explained by reverse causality. The question of causality, however, remains unanswered.

Besides CRP, there are several other markers of inflammation that can be used to study its relationship with cancer. Similarly to the NLR, the systemic immune-inflammation index (SII) is a composite marker of subtypes of the total leukocyte count. [14,26] However, in addition to the neutrophils (N) and lymphocytes (L) it also contains the absolute platelet count (P) and is calculated as followed: N/L x P. [26] Earlier it was showed that the SII outperforms the NLR in predicting mortality in patients diagnosed with cancer.

The SII is a reflection of the functioning of the immune system and could provide more insight into the role of the immune system in cancer development. We hypothesised that if immune cells play a role in the etiology of cancer, individuals with the longest exposure and follow-up should have the highest risk of cancer (**chapter 6**). The association between the SII and cancer was robust and when we explored the association over time, it appeared that the risk was increased in the first 6 months of follow-up. This could be explained by a systemic inflammatory response to a cancer that was already present but not yet detected at baseline. However, the overall effect could not merely be explained by reverse causality. The risk persisted after exclusion of data individuals with a follow-up of 6 months or less, and increased when we subsequently evaluated the risk for individuals with a follow-up period of more than 2, 5 or even 8 years of follow-up. This phenomenon may support the hypothesis that chronic inflammation is a risk factor for cancer development.

The results presented in **chapter 6** lead to the idea that maybe the SII could also be a marker of immune system impairment. A properly functioning immune system recognizes and eliminates tumour cells. However, aggressive cancers are able to evade this surveillance. [27-30] This may specifically be the situation in pancreatic cancer, that is capable of misleading the immune system in such a way that it no longer attacks tumor cells, but rather forms a support structure for the cancer. [30-32]

Pancreatic cancer is one of the most aggressive and deadliest solid tumors and is often diagnosed when the disease has already metastasized (**chapter 7**). Early detection could help improve the poor survival of this cancer.

A previous study has shown that an increased SII in patients with pancreatic cancer was associated with a poorer survival. Therefore, in **chapter 8**, we investigated whether SII levels change prior to diagnosis of pancreatic cancer. We found that SII levels were higher in patients with pancreatic cancer than in the general population, whereas initially they had comparable SII levels to the general population. Given that the results from this study were largely driven by retrospectively collected data, we should also consider the possibility that the results from this study can be explained by selection or information bias. Therefore we should be cautious to consider the SII as a marker for early detection, until these findings are reproduced and validated.

Lastly, in **chapter 9** we searched for metabolomic biomarkers that can be used for the early detection of pancreatic cancer. It is well known that the development and progression of pancreatic cancer are associated with alterations in the systemic metabolism such as glucose intolerance, anorexia and severe weight loss.[33,34] Other researchers recently found plasma metabolic markers that were increased years prior to the diagnosis of cancer.[35] However, when we set out to validate these metabolomes in five large European population-based cohorts the results could not be replicated. Therefore it is unsure whether metabolomics will be part of a successful screening program for pancreatic cancer.

Discussion and future perspectives

Methodological and statistical considerations

In any observational study there are reasons for the found association to in fact be spurious due to bias and confounding. [36] Given the prospective nature of our studies and the standardized collection of exposure and outcome, the likelihood of bias is limited. Confounding is always a potential issue, no matter how well designed the study is. However, in the Rotterdam Study we have dense information on a large number of clinically relevant and potential confounders for which we were able to adjust the association.

Special attention should be given to the confounder 'age'. As we have shown, it can be difficult to disentangle whether changes in the immune system are part of the normal ageing process or whether they are part of the physiology of disease. For the former, there may be statistical methods to account for the effect of ageing: for instance using age as an alternative time scale in a Cox proportional hazard model. [37] Generally, follow-up time in the study is used as an underlying time-metric in which age is one of the variables for which the risk estimates are adjusted. Alternatively, age can also be used as a time-scale, in which individuals of the same age at entry of the study and same amount of follow-up time in the study are directly compared with each other. [37,38] This type of analysis deals with the effect of calendar age, however does not account for what we consider as biological ageing.

Causality versus consequence

Nonetheless, even if the found association is free from bias and confounding, this does not automatically prove causality and the question remains: 'Is it inflammation that leads to the cancer or is inflammation a result of the cancer?' [36,39] In 1965, Bradford-Hill outlined nine issues to be considered when interpreting whether an association found in an observational study is explained by a causal relationship. [40,41] However it must be stressed, that these nine issues do not form criteria, and according to Bradford-Hill they were neither required nor sufficient for establishing causation. [40,41] Two of these suggestions are 'temporality 'and 'biological gradient'. In our studies we have tried to provide an insight into this by performing time-varying analyses and assessing dose-related effects.

Some would argue that the found associations are merely suggestive and provide no definitive proof that inflammation plays a causal role in cancer, because epidemiologic relations are suggestive by nature and only fundamental research can reveal true cause-effect relations. [39] However, we believe that the different areas of science: fundamental, observational or experimental results will provide complementary results that help the scientific community in establishing what is true causality.

Future perspectives

There is an increasing amount of evidence showing that risk factors such as obesity, smoking, and lack of physical activity are associated with low-grade inflammation. [42,43] The underlying assumption in the discussion whether chronic inflammation leads to cancer, is of course that it represents a reversible state and that reversing such inflammation could help decreasing cancer incidence. In this situation CRP, the NLR and SII could be considered as surrogate markers of inflammation that could be used in intervention trials to evaluate if low-grade inflammation indeed is a reversible health state.

Increased CRP, NLR or SII levels could also be seen as part of a systemic, inflammatory response to the cancer. In this instance these markers could be used to evaluate response to (immune) therapy and stratify patients who need to undergo systemic therapy. [44] Especially for cancers, such as pancreatic cancer, in which there are limited treatment options and chemotherapeutic treatments have low response rates. It would be beneficial for patients if we could prevent the morbidity and mortality associated with systemic therapy. However, it must be stressed that although the NLR and SII give more insight into the inflammatory response, than the total leukocyte count, it provides only a rough estimate. Also the neutrophils and lymphocytes have multiple sub-populations that potentially have different functions and further research is needed to elucidate their roles.

The fact that we have seen that increased levels of inflammatory markers can be measured months before patients are diagnosed with cancer, has led to the idea that they could be used for early detection. In 1968 Wilson and Junger established several ground rules for screening in the general population. [45] One of the criteria is that the test used should be effective in finding the disease. If it is not sensitive enough, it will lead to falsely believe no

cancer is present and delay proper treatment. If it is sensitive, but non-specific this might lead to over-diagnosis and unnecessary stress in patients. [45] It remains to be seen whether the NLR and SII will uphold to these standards, however given that , like CRP, they are non-specific markers of inflammation it seems unlikely that the NLR and SII, as a single factor, will be used as a test for early detection or risk stratification.

The SII can be interpreted as a direct reflection of the functioning of the immune system and not just a surrogate marker of inflammation. Increased SII levels might then be viewed as an impairment of the functioning of that immune system. That the SII was increased in pancreatic cancer is remarkable, because pancreatic cancer is considered much less immunogenic than melanoma and lung cancer. [46] However, it is known that, also in pancreatic cancer, the tumour and its environment can reprogram immune cells to neutralise its anti-tumour effects and promote inflammation. [47,48] For instance, cancer cells can promote the differentiation of monocytes into anti-inflammatory macrophages, impairing their immune response and promoting tumour growth [49,50] Novel immunotherapeutic agents are trying to address ways to revert the reprogramming of cells and trying to recover their anti-tumour properties.

The same inflammatory environment that favours the development of cancer has also been linked to homing and engraftment in peripheral tissue by bone marrow-derived cells (BMDCs). [51] Recent studies have suggested that BMDCs may possess an unexpected degree of plasticity and often home to sites of chronic injury or inflammation. [52,53] Subsequently, these cells progress through metaplasia and dysplasia to intraepithelial cancer. [51]

Lastly, chronic inflammation may also directly impair the functioning of immune cells. It has been hypothesised that innate immunity can be influenced by previous encounters with pathogens or their products, and this property has been termed "trained immunity". [54] A recent study showed that the persistent state of heightened innate immune cell activation in trained immunity, albeit beneficial in the context of recurrent infections, contributes to progression of atherosclerosis development and to acute destabilization of existing atherosclerotic plaques. [55,56]

This type of maladaptation of innate immune cells might also be translated to the local immune responsiveness of immune cells in tumour environments and contribute to an increased risk of cancer. [47,54] The trained immunity is largely driven by epigenetic reprogramming at the level of histone methylation and acetylation, and therefore the epigenetic memory of cells could potentially be used as a target for new drugs. [54,57,58]

In conclusion, in order for immunotherapy to become a part of the standard treatment regiment, future research should further elucidate the role immune cells play in the development and progression of aggressive cancers. For instance by understanding which sub-populations of lymphocytes are responsible for decreased anti-tumour immune properties, we will be able to revert pro-tumour processes and be able to improved prognosis of different cancers.

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Chapter 11

Nederlandse samenvatting

Het immuunsysteem is een verdedigingssysteem dat ons beschermt voor bedreigende pathogenen en virussen van buitenaf. Tevens beschikt het over het vermogen om beschadigde of kankercellen te detecteren en vernietigen. Naar mate we ouder worden, verandert het immuunsysteem en wordt het minder effectief in het reguleren van inflammatoire processen. Dit draagt waarschijnlijk bij aan de toename van de incidentie van morbiditeit in de verouderende populatie.

Daarbij gaat veroudering samen met een hyper-inflammatoire status. Dit wordt gekenmerkt door een verhoging van inflammatoire markers in het bloed zoals: C-reactive protein (CRP) en IL-6. Deze markers zijn geassocieerd met een verhoogd kans op morbiditeit en met mortaliteit, echter is het onduidelijk of dit het gevolg is van het normale verouderingsproces en verval van het immuunsysteem of dat het wordt veroorzaakt door onderliggende ziekten. In dat laatste geval, kunnen deze markers gezien worden als indicatoren van deze ziekten.

Het doel van dit proefschrift was om een beter begrip te krijgen van de rol van het immuunsysteem in relatie tot de ontwikkeling van kanker. Daarom hebben we inflammatoire markers onderzocht in relatie tot kanker en mortaliteit, in zowel gezonde, maar verouderende individuen, als in patiënten met kanker.

De neutrofielen-lymfocyten-ratio (NLR) is een marker die is samengesteld uit subtype cellen van het totale aantal leukocyten. De NLR wordt berekend door het absolute aantal neutrofielen te delen door het absolute aantal aan lymfocyten. De marker is ontwikkeld om de verschillende subtype cellen tegelijkertijd te kunnen bestuderen. Want, hoewel het totale aantal leukocyten geassocieerd is met mortaliteit, de verschillende subtypen: neutrofielen en lymfocyten daar een tegenovergestelde rol in lijken te spelen.

Wat de interesse in deze marker wekte, was dat het ook een weerspiegeling geeft van het functioneren van het immuunsysteem. Daarmee vormde het een interessante marker om het immuunsysteem te bestuderen in een verouderende populatie.

In **hoofdstuk 3** hebben we eerst de referentiewaarden van de NLR onderzocht. We vonden dat deze referentiewaarden niet alleen hoger waren voor mensen in een hogere leeftijdscategorie, maar ook dat de NLR binnen individuen steeg over de tijd. Dit werd veroorzaakt door zowel een afname in het aantal lymfocyten, als een toename in het aantal neutrofielen. Of de door ons geobserveerde toename van de NLR een onderdeel is van het normale verouderingsproces, of dat dit het gevolg is van onderliggende ziekten is nog onduidelijk.

In **hoofdstuk 4** lieten we zien dat individuen met een verhoogde NLR sneller kwamen te overlijden. Mogelijk is deze associatie causaal, echter is het ook mogelijk dat de NLR een surrogaat marker is voor veroudering, dan wel voor een onderliggende ziekte of een slechte gezondheidstoestand. Los van dit feit, zou de NLR een potentiële marker kunnen zijn voor risico stratificatie van patiënten in de kliniek.

De bezinking (BSE) is een bekende marker van inflammatie die veel gebruikt wordt in de kliniek. De BSE kan licht verhoogd zijn in ouderen, met name in oudere vrouwen. Er werd zelfs gezegd dat, om die reden, licht verhoogde BSE waarden gemeten in oudere patiënten buiten beschouwing konden worden gelaten. In **hoofdstuk 5** lieten we echter zien dat, ook op oudere leeftijd, een verhoogde BSE geassocieerd is met mortaliteit. Daaruit concludeerden wij dat een verhoogde BSE op oudere leeftijd niet genegeerd mag worden, maar juist vervolgd dient te worden.

Nadat we inflammatie hadden bestudeerd in haar relatie tot veroudering en mortaliteit, richtten we ons onderzoek op de rol van inflammatie in kanker.

CRP is één van de meest bestudeerde inflammatoire markers in relatie tot kanker. Echter welke rol het CRP speelt is nog altijd onduidelijk. De associatie tussen CRP en kanker kan op verschillende manieren worden verklaard. Verhoogde CRP waarden zouden direct kunnen leiden tot kanker, verhoogde CRP waarden zouden een afspiegeling kunnen zijn van aanwezige ontsteking die leidt tot de kanker, of verhoogde CRP waarden zijn het gevolg van een inflammatoire respons tegen de kanker.

Om hier een beter inzicht in te krijgen, hebben we een meta-analyse uitgevoerd van prospectieve studies die de associatie tussen CRP en incidente kankers onderzochten (**hoofdstuk 2**). We vonden een groot aantal studies van hoge kwaliteit. Echter was er dermate veel variatie in de methoden van analyseren en het rapporteren van de uitkomsten, dat slechts een klein deel kon worden meegenomen in de meta-analyse. Desalniettemin, vonden we een significante associatie tussen CRP en kanker incidentie, in het specifiek voor het longcarcinoom en het mammacarcinoom.

Een deel van de studies liet in een aanvullende analyse zien dat de associatie over de tijd iets veranderde, maar dat deze niet volledig verklaard kon worden door 'reverse-causality'. Een definitief antwoord op de vraag of het CRP een causale rol speelt in kanker, blijft echter vooralsnog uit.

Net zoals de NLR, is de systemic immune-inflammation index (SII) een inflammatoire marker die samengesteld is uit neutrofielen (N), lymfocyten (L), maar daarbij ook uit trombocyten (P). De SII wordt als volgt berekend: N/L x P. Wanneer er onderzoek gedaan wordt naar kanker, lijkt de SII een betere voorspeller te zijn dan de NLR. Ook de SII geeft een weerspiegeling van het functioneren van het immuunsysteem. Daarmee zou het bestuderen van deze marker ons meer inzicht kunnen geven in de rol van het immuunsysteem in de ontwikkeling van kanker. Onze hypothese was, dat als immuun cellen een rol zouden spelen in de etiologie van kanker, individuen met de langste blootstelling aan inflammatie ook het hoogste risico op de ontwikkeling van kanker zouden moeten hebben (**hoofdstuk 6**). De associatie tussen de SII en de diagnose van een incidente kanker was robuust. Toen we dit effect over de tijd bestudeerden, zagen we dat het risico verhoogd was in de eerste 6 maanden van follow-up. Het zou kunnen dat wat we hier zagen een inflammatoire response

is op een kanker die weliswaar al aanwezig is, maar nog niet gediagnosticeerd. Echter ook op de lange termijn bleef het verhoogde risico bestaan. Sterker nog, het risico nam ook toe over de tijd. Dit suggereert dat chronische inflammatie een rol speelt bij de ontwikkeling van kanker.

De resultaten uit **hoofdstuk 6** leidde tot de hypothese dat de SII mogelijk ook een marker zou kunnen zijn van een minder functionerend immuunsysteem. Een goed functionerend immuunsysteem herkent en vernietigd circulerende kankercellen. Echter sommige kankers kunnen deze immuno-surveillance ontwijken. Een specifiek voorbeeld hiervan is het pancreascarcinoom. Deze kanker kan het immuunsysteem zo misleiden dat het de kanker niet langer aanvalt, maar de kanker juist ondersteunt.

Daarom onderzochten we in **hoofdstuk 8** of er veranderingen waren in het immuunsysteem (gemeten door de verandering in SII waarden) van patiënten jaren voor zij de diagnose pancreascarcinoom kregen. We zagen dat SII waarden ten tijde van de diagnose veel hoger waren dan waarden in de algehele populatie, terwijl ze initieel vergelijkbaar waren. De SII leek met name te stijgen in de 2 jaar voordat de diagnose werd gesteld. Echter, de resultaten van dit onderzoek werden gedreven door retrospectief verzamelde data, dus is het mogelijk dat hier een bias in zit. Daarom zullen deze resultaten eerst moeten worden bevestigd en gevalideerd in andere studies, voordat we hier definitieve conclusies aan kunnen verbinden.

In het laatste hoofdstuk van dit proefschrift (**hoofdstuk 9**) onderzochten we of metabole markers gebruikt kunnen worden voor vroege detectie van het pancreascarcinoom. Het is bekend dat het pancreascarcinoom gepaard gaat systemische metabole veranderingen zoals, glucose-intolerantie, anorexia en ernstig gewichtsverlies. Recentelijk werden er een aantal metabole markers gevonden, die jaren voorafgaand aan de diagnose al waren toegenomen. Echter, toen wij deze metabole markers wilden valideren in vijf grote Europese cohorten, konden deze resultaten niet worden gerepliceerd. Daarom is het onduidelijk of 'metabolomics' onderdeel zullen voor van een succesvol screening onderzoek voor het pancreascarcinoom.

Appendices

Curriculum Vitae
List of Publications
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Acknowledgements

Curriculum Vitae

Jesse Fest werd geboren op 19 mei 1991 te Leidschendam. Ze groeide op in Woubrugge en Sommelsdijk – Middelharnis als oudste van een gezin van vier kinderen. Al tijdens haar middelbare schooltijd had ze belangstelling voor wetenschappelijk onderzoek en werd ze geselecteerd voor de *Junior Med School*, een extra-curriculair programma van het Erasmus MC voor middelbare scholieren. In 2009 behaalde ze haar eindexamen en tevens haar *International Baccalaureate* aan het Tweetalig VWO aan het Wolfert van Borselen in Rotterdam (cum laude). Datzelfde jaar verhuisde ze naar Rotterdam en startte zij aan de opleiding Geneeskunde aan het Erasmus MC. Tijdens haar Bachelor volgde ze het *Honours Class* programma en werkte zij in het studententeam van de kinderchirurgie in het Sophia Kinderziekenhuis.

Haar interesse voor de heelkunde werd verder aangewakkerd tijdens haar Master Geneeskunde, toen zij haar coschappen liep in het Reinier de Graaf Gasthuis in Delft en het Daniël den Hoed in Rotterdam. De Master Geneeskunde combineerde ze met een Research Master in Clinical Research, die haar opleidde tot epidemioloog. Tijdens deze Master kreeg ze de mogelijkheid om het Deutsches Krebsforschungszentrum in Heidelberg te bezoeken en deel te nemen aan het Graduate Summer Institute of Epidemiology and Biostatistics van het Johns Hopkins in Baltimore. Na haar afstuderen in mei 2016 zette ze haar onderzoek voort onder de begeleiding van prof. dr. C.H.J. van Eijck, prof. dr. B.H. Stricker en dr. R. Ruiter. Na twee jaar fulltime onderzoek, startte zij per 1 juli 2018 als art-assistent chirurgie in het Maasstad Ziekenhuis. Haar onderzoek zette zij daarnaast voort en dat werk heeft geleid tot dit proefschrift. Momenteel werkt ze als arts-assistent chirurgie in het Leids Universitair Medisch Centrum. In de toekomst hoopt ze haar klinische werk te kunnen blijven combineren met het doen van onderzoek.

List of Publications

Publications in this thesis

J. Fest, R. Ruiter, B.H. Stricker, C.H.J. van Eijck. C-reactive protein and the risk of incident cancer – a meta-analysis of prospective cohort studies. *(Submitted)*

J. Fest, R. Ruiter, M.A. Ikram, T. Voortman, C.H.J. van Eijck, B.H. Stricker. Reference values for white blood-cell-based inflammatory markers in the Rotterdam Study: a population-based prospective cohort study. *Sci Rep*. 2018 Jul 12;8(1):10566. doi: 10.1038/s41598-018-28646-w.

J. Fest, R. Ruiter, B. Groot Koerkamp, M.A. Ikram, C.H.J. van Eijck, B.H. Stricker. The neutrophil-to-lymphocyte ratio is associated with mortality in the general population: results from the Rotterdam Study. *Eur J Epidemiol*. 2019 May;34(5):463-470. doi: 10.1007/s10654-018-0472-y. Epub 2018 Dec 19.

J. Fest, R. Ruiter, S.P. Mooijaart, M.A. Ikram, C.H.J. van Eijck, B.H. Stricker. Erythrocyte sedimentation rate as an independent prognostic marker for mortality: a prospective population-based cohort study. *J Intern Med*. 2019 Mar;285(3):341-348. doi: 10.1111/joim.12853. Epub 2018 Dec 9.

J. Fest, R. Ruiter, M. Mulder, B. Groot Koerkamp, M.A. Ikram, B.H. Stricker, C.H.J. van Eijck. The systemic immune-inflammation index and risk of cancer in a population-based cohort study. *Int J Cancer.* 2019 Mar 28. doi: 10.1002/ijc.32303. [Epub ahead of print]

J. Fest, R. Ruiter, F.J.A. van Rooij, L.G.M. van der Geest, V.E.P.P. Lemmens, M.A. Ikram, J.W. Coebergh B.H. Stricker, C.H.J. van Eijck. Underestimation of pancreatic cancer in the national cancer registry - Reconsidering the incidence and survival rates. *Eur J Cancer*. 2017 Feb;72:186-191. doi:10.1016/ j.ejca.2016.11.026. Epub 2016 Dec 26.

J. Fest, R. Ruiter, M.A. Ikram, B.H. Stricker, C.H.J. van Eijck. The systemic immune-inflammation index as a marker for the impairment of the immune system in pancreatic cancer prior to diagnosis. *(In preparation)*

J.Fest, L.S. Vijfhuizen, J. J. Goeman, O. Veth, A. Joensuu, M. Perola, S. Männistö, E. Ness-Jensen, K. Hveem, T. Haller, N. Tonisson, K. Mikkel, A. Metspalu, C.M. van Duijn, M.A.Ikram, B.H. Stricker, R. Ruiter, C.H.J. van Eijck, G.B. van Ommen, P.A.C. 't Hoen.

Search for early pancreatic cancer blood biomarkers in five European prospective population biobanks using metabolomics. *Endocrinology*. 2019 May 24. doi: 10.1210/en.2019-00165. 2019 Jul 1;160(7):1731-1742. doi: 10.1210/en.2019-00165

Other publications

K.D. van der Willik, L. Fani, D. Rizopoulos, S. Licher, **J. Fest**, S.B. Schagen, M.K. Ikram, M.A. Ikram. Balance between innate versus adaptive immune system and the risk of dementia: a population-based cohort study. *J Neuroinflammation*. 2019 Mar 30;16(1):68. doi: 10.1186/s12974-019-1454-z.

M. Frelinghuysen, **J. Fest**, N.C. van der Voort- van Zyp, B. van der Holt, M. Hoogeman, J. Nuyttens. Consequences of referral time and volume doubling time in inoperable patients with early stage lungcancer. *Clin Lung Cancer*. 2017 Nov;18(6):e403-e409. doi: 10.1016/j.cllc.2017.05.002. Epub 2017 May10.

J. Fest, A. van den Boom, R. de Krijger, D. Roos. Een appendectomie gevolgd door een pancreaticoduodenectomie: solide pseudopapillair neoplasma van het pancreas. *Ned Tijdschr Geneeskd*. 2015;159: A8620.

PhD Portfolio

Research skills		**ECTS**
2012-2016	Master of Science in Clinical Research, Netherlands Institute for Health Sciences (NIHES), Erasmus University Rotterdam, the Netherlands. *Including a summer programme at Johns Hopkins, Bloomberg School of Public Health, Baltimore, USA.*	120
Presentations		
2016	JAK-STAT signalling in pancreatic cancer. AACR Special Conference on Pancreatic Cancer, Orlando. – *Poster presentation*	1
2017	Underestimation of pancreatic cancer in the national cancer registry – reconsidering the incidence and survival rates. Wetenschapsdag Heelkunde Erasmus MC, Rotterdam. – *Oral presentation*	1
	WCRF and AICR recommendations on cancer prevention and risk for pancreatic cancer. Nederlandse Vereniging voor Gastro-Enterologie voorjaarsvergadering, Veldhoven. – *Oral presentation*	1
	Underestimation of pancreatic cancer in the national cancer registry. Annual Pancreas Club Meeting, Chicago. – *Poster presentation*	1
	WCRF and AICR recommendations on cancer prevention and risk for pancreatic cancer. Annual Pancreas Club Meeting, Chicago. – *Poster presentation*	1
	WCRF and AICR recommendations on cancer prevention and risk for pancreatic cancer. Union of European Gastroenterology Week, Barcelona. – *Oral Presentation*	1
2018	The systemic immune-inflammation index and risk of incident cancer in the general population. Wetenschapsdag Heelkunde Erasmus MC, Rotterdam. – *Oral Presentation*	1
	The systemic immune-inflammation index and risk of incident cancer in the general population. MolMed Day, Rotterdam. – *Elevator Pitch*	1
	The systemic immune-inflammation index and risk of incident cancer in the general population. Nederlandse Vereniging voor Gastro-Enterologie voorjaarsvergadering, Veldhoven. – *Oral presentation*	1
	The systemic immune-inflammation index as a marker for early detection in pancreatic cancer. Annual Pancreas Club Meeting, Washington DC. – *Oral Presentation*	1
2019	Incidentie pancreascarcinoom. SWPCC congres, Rotterdam - *Oral Presentation*	1

Conferences and seminars		
2016	Wetenschapsdag Heelkunde	0.3
	AACR Special Conference on Pancreatic Cancer Orlando	1
	NVGE voorjaarsvergadering	1
	Chirurgendagen	1
2017	Wetenschapsdag Heelkunde	0.3
	NVGE voorjaarsvergadering	1
	Chirurgendagen	1
	Pancreas Club Annual Meeting Chicago	1
	Liver Metastasis Research Network Conference	1
	UEG Week Barcelona	1
2018	Wetenschapsdag Heelkunde	0.3
	MolMed Day	0.3
	Digestive Disease Days	1
	Pancreas Club Annual Meeting Washington DC	1
	ASCO Chicago	1
2019	SWPCC Congres	0.3

Teaching activities		
2016 – 2018	BLS examinations	0.75
2016 – 2019	*Junior* Medical School: selection, teaching, organisation	6.75
2017 – 2018	NIHES Pharmaco-Epidemiology course, teaching assistent	0.5
2018	Supervision Master students Statistics LUMC	1

Miscellaneous		
2017 – 2018	Peer reviewing for a variety of medical journals.	3.0

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Kamertje Z-839: tussen de stapels stoffige papieren, uitgedroogde planten en eindeloos veel gestickerde bioptpotjes, lieten jullie precies voldoende ruimte op de vensterbank over. Lieve, lieve Ing, cappuccino? Binnenkort is het weer tijd voor kaasfondue! Bo, yo, yo, yo!

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