

# **Are circulating immune cells a determinant of pancreatic cancer risk? A prospective study using epigenetic cell count measures**

Running title: Immune cells and pancreatic cancer risk

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## 1   **Abstract**

2   Background: Evidence is accumulating that immune cells play a prominent role in pancreatic cancer  
3   aetiology but prospective investigations are missing.

4   Methods: We conducted a nested case-control study within the European Prospective Investigation  
5   into Cancer and Nutrition (EPIC) study with 502 pairs of incident pancreatic cancer cases and matched  
6   controls. Relative counts of circulating immune cells (neutrophils and lymphocyte sub-lineages: total  
7   CD3+, CD8+, CD4+, and FOXP3+ regulatory T cells (Tregs) relative to nucleated cells, (white blood cells)  
8   were measured by qRT-PCR. Odds ratios with 95% Confidence Intervals were estimated using logistic  
9   regressions, modelling relative counts of immune cells on a continuous scale.

10   Results: Neither relative counts of immune cell types taken individually, nor mutually adjusted for each  
11   other were associated with pancreatic cancer risks. However, in sub-group analyses by strata of lag-  
12   time, higher relative counts of Tregs and lower relative counts of CD8+ were significantly associated  
13   with an increased pancreatic cancer risks in participants diagnosed within the first 5 years of follow-  
14   up.

15   Conclusion: These results might reflect reverse causation, due to higher relative counts of Tregs and  
16   lower counts of CD8+ cells among individuals with more advanced stages of latent pancreatic cancer,  
17   who are closer to the point of developing clinical manifest disease.

18   Impact: We have shown, for the first time, that increased relative counts of regulatory T-cells and lower  
19   relative counts of CD8+, cytotoxic T cells may be associated with pancreatic cancer risk or relatively  
20   late-stage tumor development.

## Background

Animal experiments and clinical studies have shown that innate and adaptive immune responses (immuno-surveillance) play a key role in pancreatic cancer development (1-3). The formation of pancreatic precursor lesions and the further development into an invasive tumour is accompanied by progressive infiltration of various types of immune cells (4). In pancreatic cancer patients the total or relative counts of various types of immune cells in tumour tissue (5,6), but also in peripheral blood (7,8), have been found to correlate with clinical outcomes. In general, higher total or relative counts of CD8+ cytotoxic T-cells, CD4+ T-helper 1 cells, and natural killer cells have been associated with more favourable patient outcomes, whereas higher counts of CD4+ T-helper 2 cells and of FOXP3+ regulatory T-cells have been associated with greater immunosuppression, accelerated cancer development and worse prognosis (9). In addition, neutrophil counts have often been found to be higher among cancer patients, including pancreatic cancer patients (10), as compared to cancer-free control subjects, and elevated pre-treatment ratios of circulating neutrophil-to-lymphocyte (NLR) have been associated with lower overall patient survival (11,12). However, while substantial evidence now documents the prognostic significance of immune defence among patients, so far no studies have examined whether precisely quantified immune cell homeostasis in initially healthy individuals also determines future pancreatic cancer risk. The major reason for this latter deficit is that blood samples stored in large-scale population cohort studies usually do not contain intact blood cells, prohibiting flow cytometry analyses of immune cell composition.

Recently, we have developed and validated methods that allow the determination of immune cell composition relative to total nucleated cells by quantitative PCR of epigenetic markers specific for different immune cell lineages (13). This type of assay can be applied to DNA extracted from frozen, non-intact white blood cells as well as other tissues. By applying this method to stored buffy coat samples of the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heidelberg cohort, we found that higher counts of FOXP3+ (regulatory) T-cell relative to total nucleated cells were associated with higher risks of cancers of the lung, colorectum and breast, whereas lower relative CD8+

(cytotoxic) T-cells counts were inversely associated with lung and breast cancer risks (14). By contrast, this first study showed no associations of cancer risk with relative counts of monocytes, B-cells or natural killer cells, and only marginally suggestive associations (non-significant) for neutrophils.

To explore whether similar prospective relationships exist between counts of circulating immune cells relative to total nucleated cells and risk of pancreatic cancer, overall or depending on the prospective lag-time between blood sampling (time of immune cell counts) and cancer diagnosis, we performed a case-control study nested within the Europe-wide EPIC cohort (15), with a focus on estimated relative counts for neutrophils and lymphocyte T-cell sub-lineages.

## **Study Population and Methods**

### *Study Population*

The current investigation was based on a case-control study nested within the European EPIC cohort – a multicentre prospective study in Europe described in detail previously (15). Briefly, 519,978 healthy men and women, mostly aged between 35 and 70 years, were recruited by 23 collaborating centres in 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom) between 1992 and 2000. At baseline, participants filled in comprehensive questionnaires on lifestyle, nutrition and diseases and their height, weight and body circumferences (waist and hip) were measured. In addition, study participants provided blood samples, which were processed into serum, plasma and red blood cells, as well as a buffy coat containing all nucleated cells. Processed blood cell fractions were frozen and stored centrally at the International Agency for Research on Cancer (IARC, Lyon, France), as well as locally in each recruitment centre. The EPIC centres in Malmö (Sweden) and Greece did not contribute to the present study.

Incident cancer cases were identified by population cancer registries (Denmark, Italy, the Netherlands, Spain, Sweden, and the United Kingdom) or by a combination of methods including linkage with cancer and pathology registries and systemic verification of active follow-up (France, Germany). In all EPIC centres, data on vital status were collected through population registries, in combination with national health insurance data (France).

#### *Nested Case-Control Design*

The present project is based on incident pancreatic cancer cases diagnosed between 1993 and 2008.

Pancreatic cancer incidence data were coded according to ICD-10 and included all invasive exocrine pancreatic cancers that were coded as C25 (25.0-25.3, 25.7-25.9). Exclusion criteria were the occurrence of other malignant tumours preceding the diagnosis of pancreatic cancer, except for non-melanoma skin cancer, and non-availability of blood specimens. Seventy-three percent were adenocarcinomas whereas the other 27% were not otherwise specified. Of these 145 unspecific cases, 11% were of advanced and 74% of unknown stage. Microscopically confirmed were 391 cases (78%), while the diagnosis of the remaining 22% was based on clinical observations, imaging or autopsy.

For each case subject, one control subject was selected using an incidence density sampling protocol. Case and control subjects were matched on study recruitment centre, sex, age at blood collection ( $\pm 6$  months), date of blood collection ( $\pm 2$  month), time of blood collection ( $\pm 2$  hours), length of follow-up, and time between blood sampling and time of last consumption of any foods or drinks ( $< 3$ ,  $3-6$ ,  $\geq 6$  hours). We included initially 530 incident pancreatic cancer cases and 533 controls, building 502 matched case-control pairs and 59 single subjects (31 controls, 28 cases) with no matching partner due to depleted blood samples in the biorepositories.

#### *Laboratory methods: quantification of immune cells*



Relative counts of neutrophils and lymphocyte sub-populations were measured by quantitative epigenetic real-time PCR at EPIONTIS GmbH (Berlin, Germany) (13).

DNA was extracted at the IARC, from frozen pellets of nucleated blood cells (buffy coats) using the Autopure LS DNA preparation platform (Autogen, Holliston, USA) (16). Relative counts of immune cells in genomic DNA extracted from buffy coats were assessed by quantitative real-time PCR assisted cell counting (qPACC). Descriptions of the laboratory methods are given on the Epiontis web site (<http://www.epiontis.com>), have been previously published (13,14,17-19), and are detailed in the **Supplementary Methods and Materials M1 to M4**. In brief, for each subject, 1.5µg of genomic DNA bisulfite treated prior to qPCR analysis. Relative counts of neutrophils, and lymphocyte sub-lineages (Tregs, CD4+ and CD8+ T cells) were assessed by qPACC. The assays are based on the measurement of genetic loci that have been shown to be specifically unmethylated in the target cell type, whereas methylated in all other nucleated cells of the blood. Specific gene loci (amplicon regions) used for the epigenetic cell count quantification include CD3G/CD3D (all CD3+ T cells), CD8B (CD3+/CD8+ T cells), CD4 CD4+ T cells), FOXP3 (CD3+/CD4+/FOXP3+ Tregs), and LCN2 (CD15+ neutrophils). The quantitative PCR signals for each of the immune cell types were normalized relative to the number of nucleated (white blood) cells by using a constitutively unmethylated locus in the GAPDH gene. Each of the assays has been extensively validated in various studies by comparison against absolute cell counts by flow cytometry and show very high correlations (0.85 and higher), both for absolute cells counts (per mm<sup>3</sup> of whole blood) and for relative counts as a proportion of total nucleated cells (in whole blood) (13,20).

### *Statistical Analyses*

Each individual cell lineage measured was expressed as a relative percentage of total nucleated cells in the circulation. In addition, for each study participant, the percentages for the CD4+ and CD8+ T cell sub-fractions were further normalized (re-calibrated) so as to add up to the fraction of total CD3+ T cells, and the percentages of FOXP3+ and FOXP3- T helper cells were calculated as fractions of the re-calibrated percentage of total CD4+ cells (see also **Supplementary Figure S1**, additional description in

**Supplementary Methods and Materials M1 – M4** and in (14)). The latter, hierarchical recalibration of T-cell sub-fractions allows an evaluation of improvement in model fit upon stepwise decomposition of the total CD3+ T cells into CD4+ and CD8+ sub-lineages, and of CD4+ into FOXP3+ and FOXP3- sub-lineages, as discussed below.

Multiple imputation was used to impute relative counts of immune cells missing at random in complete matched case sets, i.e. neutrophils (proportion of missing 11.1%), CD3+ (1.7%), CD8+ (1.8%), CD4+ (2.7%), FOXP3+ (5.5%), adjusted for smoking status (ever vs. never), body mass index (BMI, kg/m<sup>2</sup>), diabetes (yes, no, unknown) and case/control status. This allowed us to perform analyses in 502 matched case-control sets instead of 416 sets with originally complete immune cell data. The Markov Chain Monte Carlo method for arbitrary missing patterns was used to generate 25 imputed datasets and results were combined to generate valid statistical inferences (21). Comparable results were obtained between imputed and non-imputed datasets, albeit number of case sets were different in the two analyses and risk estimates not significant (**Supplementary Table S2**).

To examine cross-sectional relationships between relative counts of immune cells and selected baseline risk covariates in controls, partial correlations were calculated adjusted for age at blood draw and sex, for each of the 25 imputed dataset. Resulting correlation coefficients were combined using Fisher's z transformation as explained in the SAS documentation PROC MIANALYZE. Additionally, a generalised linear model (GLM) was performed to estimate the association between immune markers and diabetes risk in controls.

Conditional logistic regression models were used to examine associations of relative immune cell counts with pancreatic cancer risk, modelling immune cells as continuous variables. Statistical analyses focused in parallel on: (i), the association of pancreatic cancer risk with relative neutrophil counts; and (ii), the association of pancreatic cancer risk with relative blood counts of total (CD3+) T cells and T cell sub-fractions (CD4+ vs CD8+, and within the CD4+ fraction FOXP3+ vs other CD4 helper cells). Risk associations were examined for neutrophils and overall CD3+ T cells (Model 1), as well as in a series of

nested models. Stepwise de-composition models were fitted within the re-calibrated T-cells, breaking down total T-lymphocytes (CD3+) into its CD8+ (cytotoxic) and CD4+ (helper cell) sub-fractions (Model 2), and CD4+ into its FOXP3+ (regulatory) and FOXP3- (non-regulatory) sub-fractions (Model 3) (14). In this stepwise de-composition approach, log-likelihood ratio tests were used to examine improvements in overall model fit. As the variables for subcomponents always add up precisely to those for the total of higher-order T-cell lineages (i.e., CD4+ plus CD8+ equals total CD3+, and FOXP3+ plus FOXP3- equals CD4+), models within this two-step decomposition hierarchy can be considered nested, and stepwise improvements in model fit indicate whether, or not, subcomponent lineages have identical associations with cancer risk as compared to their higher-order sum. For example, if the model including separate variables for CD4+ and CD8+ cells shows better fit than a model including a variable only for CD3+ cells (the sum of CD4+ and CD8+) this implies that pancreatic risk has significantly different associations with CD4+ and with CD8+, and that these two risk associations are not summarized well by association of pancreatic cancer risk with the single variable of total CD3+ cells.

Analyses were performed for the full, overall case-control sets as well as by strata of lag-time (i.e. strata of 5-years follow-up) between blood draw and cancer diagnosis (< 5, 5-10 and ≥ 10 years). Heterogeneity of the associations of pancreatic cancer risk with immune cell counts by strata of prospective follow-up time was assessed as statistical interaction effect, using likelihood ratio tests. Additional adjustments for potential confounders including smoking status (never, former, current), BMI (kg/m<sup>2</sup>), self-reported pre-existing diabetes at baseline (yes/no), and alcohol lifetime intake (g/d) were examined separately in each model. Although some potential confounders were associated with pancreatic cancer risk (BMI, smoking, diabetes), none were related to immune cell counts and none of the potential confounders changed risk estimates by more than 10% and, therefore, we discarded them from our main models. Odds Ratios adjusted for lifestyle factors are very similar and shown in **Supplementary Table S3**. Sensitivity analyses were performed in microscopically confirmed cases only (n=391, 78% of cases) and by modelling lag-time in shorter intervals close to baseline (i.e. ≤2 years and 2-5yrs).

All statistical analyses and imputations were conducted using the Statistical Analysis System (SAS) software package, version 9.4 (SAS Institute Inc).

## Results

Baseline characteristics of the nested case-control participants are displayed in **Table 1**. Pancreatic cancer cases were on average 64 years old at diagnosis (range: 37 to 87) with a median follow-up of 8 years (range: 0 to 16) after baseline blood sampling. More than half of case and control subjects were overweight at recruitment (62% of cases and 55% of controls). Compared to control subjects, cases more often reported current smoking (32 vs 23%) and pre-existing diabetes (6 vs 4%) at the time of recruitment. Cases and controls showed no differences in alcohol intake (g/d) at recruitment in either men (15% of cases and 14% of controls) or women (3% in cases and controls).

Among the control subjects, on average the neutrophils constituted 54 per cent (range: 4-97) of all nucleated cells, whereas the CD3+ cells represented about 22 per cent (6-54). Within the CD3+ T-cell compartment, 15% and 7% of the total nucleated cells were CD4+ and CD8+ cells, respectively, and within the CD4+ compartment 1% of total nucleated cells were FOXP3+. Percentages were similar among the cases (**Table 1**). Adjusting for age at blood draw and sex, the relative counts of total CD3+ T-lymphocytes and all T-cell sub-fractions (CD8+, CD4+, FOXP3+) were inversely correlated with neutrophils ( $r \leq -0.40$ ) in controls. Within the T-cell lineage, there was a moderate correlation ( $r=0.51$ ) between the relative counts of CD8+ T and CD4+ T-cells, and a positive correlation ( $r=0.65$ ) was also observed between relative counts of FOXP3+ and total CD4+ cells. All cell types showed only weak and non-significant correlations ( $r < 0.16$ ) with BMI, waist circumference, smoking (pack years), time from quitting smoking or alcohol consumption (**Figure 1**). Additionally using GLM, the average of relative counts of immune cells were not statistically different between diabetes status (e.g. neutrophils  $p=0.21$ , CD3+  $p=0.83$ , CD8+  $p=0.78$ , CD4+  $p=0.90$ , FOXP3+  $p=0.89$ ).

Regarding associations with pancreatic cancer risk, conditional logistic regression showed no significant associations per percent unit increase of neutrophils or total CD3+ T-lymphocytes with pancreatic cancer, irrespectively of the lag time between blood donation and cancer diagnosis (**Table 2**). Likewise, in the full case-control study set there were no significant improvements in the overall fit for multivariable models when the counts for overall CD3+ T-cells (model M1) were broken down into the constituent counts for cytotoxic (CD8+) T-lymphocytes and CD4+ (helper) T cells (model M2), or when counts of the CD4+ compartment were further broken down into regulatory (FOXP3+) and non-regulatory (FOXP3-) T-lymphocytes (model M3). In analyses by strata of lag-time, however, the hierarchical decomposition into T-cell constituents resulted in borderline significant improvement of model fit when focusing on pancreatic cancer cases whose blood samples were collected less than 5 years prior to cancer diagnosis ( $p_{M2M3}=0.05$  or  $p_{M1M3}=0.03$ ). Within this 0-5 year lag-time interval, the decomposed, mutually adjusted model showed higher pancreatic cancer risk for higher relative counts of regulatory (FOXP3+) T-lymphocytes (for a percent unit: OR=1.80; 95%CI=1.01-3.20), and for lower counts of cytotoxic CD8+ lymphocytes (OR=0.92; 95%CI=0.85-1.00) (**Table 2**). For lag-times between blood donation and cancer diagnosis longer than 5 years these associations and improvements in overall model fit were not observed. Above observed risks were stronger in analyses restricted to microscopically confirmed cases, i.e. OR=2.22 (95% CI=1.12-4.40) for higher FOXP3 and OR=0.92 (95% CI=0.83-1.02) for higher CD8+ within the first five years of follow-up ( $p_{M2M3}=0.01$  or  $p_{M1M3}=0.02$ ) (not tabled). Further detailed lag-time analyses showed step-wise higher non-significant associations for FOXP3 the shorter the follow-up, i.e. the closer to baseline ( $\leq 2$  yrs OR=2.15; 95% CI=0.55-8.40 with  $n_{cases}=52$ , 2-5yrs OR=1.74 95% CI=0.92-3.31 with  $n_{cases}=95$ , and 5-10yrs OR=1.06 95%CI=0.69-1.63; data not in tables). Significant Likelihood ratio tests were observed for the interaction between length of follow-up and relative immune cell counts of FOXP3+ across follow-up categories (<5yrs vs. 5-10yrs  $p=0.04$ ).

No significant associations were observed in analyses stratified by smoking status (ever/never) (not tabled). Further stratification by follow-up, however, did show higher pancreatic cancer risk with

higher FOXP3+ in ever smokers (for a percent unit: OR=1.87 95%CI=1.05-3.33) with follow-up <5 years, although not significant in fully adjusted models.

Additional adjustments for relative counts of neutrophils did not materially alter the fit of any of the above models or alter any of the estimated associations (logistic regression coefficients) between relative cell counts and pancreatic cancer risk.

## Discussion

Using a nested case-control design within the European EPIC cohort, we examined the prospective relationship of relative immune cell counts in blood of initially healthy individuals with pancreatic cancer risk, focusing on neutrophils and total (CD3+), cytotoxic (CD8+), and regulatory (FOXP3+) and non-regulatory (FOXP3-) CD4+ (helper) T-lymphocytes. To determine the relative quantities of these cell types we used quantitative real-time PCR assays of DNA methylation markers, which provide highly accurate measurements as validated by comparison to absolute to relative cell counts by flow cytometry (13).

The adaptive immune response among healthy individuals depends on the balance between cytotoxic CD8+ effector T cells, which drive the elimination of abnormal cells, and FOXP3+ regulatory T lymphocytes (Tregs), which modulate the aggressiveness of the cellular immune response (22,23). In patients with pancreatic cancer, studies have found greater tumor aggressiveness and worse survival when tumor tissue (especially the tumor center) showed higher FOXP3+ T cell infiltration, whereas high infiltration of particularly CD8+ T cells generally corresponded with better outcomes (24,25). Similarly, studies have shown worse oncologic outcomes, e.g. cancer-specific survival including pancreatic cancer (9), among patients who present with higher Tregs and lower CD8+ T-lymphocyte counts in blood (26-28). It thus appears plausible that, also among initially healthy individuals, lower

relative blood cell counts of CD8+ effector T-lymphocytes and higher counts of Tregs within the CD4+ T helper cell compartment may be related to higher risk of future cancer development.

Contrary to our expectations, our present study shows no long-term associations of pancreatic cancer risk with the relative quantities of CD8+, or FOXP3+ and FOXP3- (CD4+) T cell components, when we used statistical models with stepwise decomposition of total (CD3+) T cells into cytotoxic (CD8+), regulatory (FOXP3+), and non-regulatory (FOXP3-) helper cells. These overall null findings stand in contrast to our earlier results from the EPIC-Heidelberg cohort, where over prospective follow-up times up to 15 years (median 6.7 years) this step-wise modeling approach revealed a higher long-term risk of cancers (lung, breast) among initially healthy individuals who had lower proportions of CD8+ T cells within the overall T-cell compartment, or who had higher proportions of FOXP3+ regulatory T cells among the total circulating CD4+ helper T cells (cancers of the lung, breast, and colorectum) (14). However, in subgroup analyses, considering blood samples collected no more than 5 years prior to cancer diagnosis, our present findings did suggest an association of pancreatic cancer risk with a T-lymphocyte signature similar to that identified in our previous EPIC-Heidelberg study, namely higher pancreatic cancer risk among those who had higher relative counts for FOXP3+ regulatory T cells among the total CD4+ helper T cells, and lower proportions of cytotoxic (CD8+) T-lymphocytes. For pancreatic cancer patients whose blood samples had been collected more than 5 years prior to diagnosis, and their matched control subjects, this association pattern was not observed.

The most plausible explanation for seeing associations between relative immune cell counts and pancreatic cancer risk only in the first five years of follow-up is reverse causation, the associations reflecting progressive increases in circulating Tregs, and decreases in CD8+ cells, as future pancreatic cancer patients gradually develop more advanced-stage and eventually symptomatic tumors. The well-described model of pancreatic cancer development via precursor lesions (29) and massive infiltration and later shedding of immune cells to the circulation (3) may also support this latter interpretation. Unfortunately, we could not examine whether the associations of immune cell composition with pancreatic cancer within the first 5 years of prospective follow-up varied according to stage at

270 diagnosis, due to incomplete information on tumor stage. Most pancreatic cancers, however, are  
271 diagnosed in advanced stage (30).

272 Neutrophils were originally considered to have pro-inflammatory functions as part of the innate  
273 immune responses and as effectors of acute inflammation, but are increasingly being recognized to  
274 also exert a broader array of specialized functions in adaptive immune reactions and chronic  
275 inflammatory responses to cancer and other diseases (31,32). In blood, patients with various types of  
276 solid tumors, including pancreatic cancer (10,33,34), often show increased neutrophil counts  
277 compared to cancer-free control subjects, and higher ratios of circulating neutrophils to lymphocytes  
278 (NLR) have been associated with more advanced disease stage and poorer cancer-specific survival  
279 rates (35,36). Again, however, contrary to our initial expectations our present data show no significant  
280 association of pre-diagnosis relative counts of peripheral neutrophils with risk of developing pancreatic  
281 cancer. It is worth noting that, in our previous study in the EPIC-Heidelberg cohort, we also did not  
282 identify any prospective association of relative neutrophil counts with risks of cancers of the lung,  
283 breast, colorectum or prostate (14).

284 To our knowledge, our analyses are the first to relate pancreatic cancer risk to relative measures of T-  
285 lymphocyte composition using a biologically validated set of cell lineage-specific epigenetic markers,  
286 in individuals initially free of known cancer. Cell proportions estimated from epigenetic cell lineage  
287 markers – derived from methylation arrays – are nowadays more frequently used. While this chip array  
288 technology is functionally not associated and the role and association of individual CpGs is mostly  
289 unknown, these approaches do confirm our currently used approach. Michaud and colleagues recently  
290 published on DNA methylation derived immune cell profiles and pancreatic cancer risk but did not  
291 observe any association with CD3+, CD4+ and neutrophils, amongst others, irrespective of lag-time  
292 (37). Their method differs from ours, and used deconvolution algorithms to estimate relative immune  
293 cell counts from a broader series of epigenome-wide methylation markers, whereas our more precise  
294 and more powerful method uses not only cell type associated but also co-methylated regions that  
295 were biologically valid and extensively validated against flow cytometry (13).



A limitation of our study is that the epigenetic assays for buffy coat samples allowed quantification only of relative immune cell counts, relative either to total nucleated cells or to a higher-order cell lineage for T-cell compartments, but not of absolute cell counts relative to blood volume. Nonetheless, relative counts by our epigenetic assays show excellent correlations ( $r \geq 0.85$ ) with counts obtained by flow cytometry (13). Another limitation is that only a single, baseline blood sample is available for the EPIC cohort participants. In our previous study in the EPIC-Heidelberg cohort, however, we found that there was a relatively high intra-individual stability of relative immune cell counts between repeat blood samples collected over time for a sub-set of study participants, with age- and sex-adjusted partial Spearman correlations over a 15-year time interval of about 0.50 for neutrophils, total CD3+, total CD4+ and FOXP3+ cells, and of 0.67 for CD8+ cells (14).

In summary, this study in initially cancer-free individuals showed no long-term relationship between blood counts of neutrophils or T-lymphocyte sub-lineages with later risk of developing pancreatic cancer. However, within prospective follow-up times of less than 5 years between blood sampling and cancer diagnosis, our data suggest a possible higher pancreatic cancer risk in relation to circulating immune cell signature characterized by higher Treg-mediated immune tolerance, and lower CD8+ mediated cytotoxicity – a signature that we previously found to be associated with higher risks of cancers of the lung, breast and colorectum. These time-restricted associations may reflect reverse causation – i.e. alterations in circulating immune cell composition induced that result from progressive pancreatic cancer development.

To our knowledge, these analyses are the first to relate pancreatic cancer risk to specific and well-validated epigenetic markers as quantitative measures of circulating T-lymphocyte composition and neutrophil counts in individuals initially free of known cancer. Further studies will be needed to confirm our observations and to assess whether, at all, increased Treg-mediated immune tolerance or reduced CD8+ mediated cytotoxicity have any impact on pancreatic cancer risk, and if so, whether this impact is mostly on relatively late-stage tumor development, that is, within relatively short lag-times between blood donation and cancer diagnosis. If further epidemiologic studies confirm findings and show a

322 consistent pattern of associations between circulating immune cell composition and cancer risk, we  
323 hope this might open up novel avenues for cancer prevention, focusing on ways to optimize  
324 individuals' general immune defense.

325	CI	confidence interval
326	CD3+	overall T cells
327	CD4+	helper T cell
328	CD8+	cytotoxic T cell
329	FOXP3+regulatory (CD4+) T helper cell (Treg).	
330	EPIC	European Prospective Investigation into Cancer and Nutrition
331	IARC	International Agency for Research on Cancer
332	ICD	International Classification of Diseases
333	OR	Odds Ratio
334	Treg	regulatory T cell

335

336

### 337 *Additional Information*

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344

### 345 *Authors' contributions*

R.K. and V.K. conceived and designed the study. V.K. organized the study. S.O. coordinated the laboratory analyses. V.K. and C.LC pre-processed and analyzed the data and V.K., C.LC, and R.K. wrote the first version of this manuscript. All authors critically revised and approved the manuscript.

#### *Ethics approval and consent to participate*

This research project has been performed in accordance with the Declaration of Helsinki and has been approved by the Ethical Committee at IARC (IEC 14-01) and the Medical Faculty Heidelberg (S-103/2015).

#### *Consent for publication*

All authors consent publication.

#### *Data availability*

The EPIC project was launched in the 1990s. Unlike in new studies that we run today, public access to data from the EPIC population was not part of the study protocol at that time. Thus, the data protection statement and informed consent of the EPIC participants do not cover the provision of data in public repositories. Nevertheless, we are open to providing our dataset upon request for (a) statistical validation by reviewers and (b) pooling projects under clearly defined and secure conditions and based on valid data transfer agreements.

#### *Conflict of interest*

The authors declare no conflicts of interest.

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## *Disclaimer*

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- 396 1. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, Vonderheide RH. Dynamics of the  
397 immune reaction to pancreatic cancer from inception to invasion. *Cancer Res*  
398 **2007**;67(19):9518-27 doi 10.1158/0008-5472.CAN-07-0175.
- 399 2. Huber M, Brehm CU, Gress TM, Buchholz M, Alashkar Alhamwe B, von Strandmann EP, *et al.*  
400 The Immune Microenvironment in Pancreatic Cancer. *International journal of molecular*  
401 *sciences* **2020**;21(19) doi 10.3390/ijms21197307.
- 402 3. Inman KS, Francis AA, Murray NR. Complex role for the immune system in initiation and  
403 progression of pancreatic cancer. *World journal of gastroenterology : WJG* **2014**;20(32):11160-  
404 81 doi 10.3748/wjg.v20.i32.11160.
- 405 4. Karamitopoulou E. Tumour microenvironment of pancreatic cancer: immune landscape is  
406 dictated by molecular and histopathological features. *Br J Cancer* **2019**;121(1):5-14 doi  
407 10.1038/s41416-019-0479-5.
- 408 5. Fukunaga A, Miyamoto M, Cho Y, Murakami S, Kawarada Y, Oshikiri T, *et al.* CD8+ tumor-  
409 infiltrating lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells  
410 improve the prognosis of patients with pancreatic adenocarcinoma. *Pancreas* **2004**;28(1):e26-  
411 31.
- 412 6. Ino Y, Yamazaki-Itoh R, Shimada K, Iwasaki M, Kosuge T, Kanai Y, *et al.* Immune cell infiltration  
413 as an indicator of the immune microenvironment of pancreatic cancer. *Br J Cancer*  
414 **2013**;108(4):914-23 doi 10.1038/bjc.2013.32.
- 415 7. Ikemoto T, Yamaguchi T, Morine Y, Imura S, Soejima Y, Fujii M, *et al.* Clinical roles of increased  
416 populations of Foxp3+CD4+ T cells in peripheral blood from advanced pancreatic cancer  
417 patients. *Pancreas* **2006**;33(4):386-90 doi 10.1097/01.mpa.0000240275.68279.13.
- 418 8. Yamamoto T, Yanagimoto H, Satoi S, Toyokawa H, Hirooka S, Yamaki S, *et al.* Circulating  
419 CD4+CD25+ regulatory T cells in patients with pancreatic cancer. *Pancreas* **2012**;41(3):409-15  
420 doi 10.1097/MPA.0b013e3182373a66.
- 421 9. Karakhanova S, Ryschich E, Mosl B, Harig S, Jager D, Schmidt J, *et al.* Prognostic and predictive  
422 value of immunological parameters for chemoradioimmunotherapy in patients with  
423 pancreatic adenocarcinoma. *Br J Cancer* **2015**;112(6):1027-36 doi 10.1038/bjc.2015.72.
- 424 10. Arima K, Okabe H, Hashimoto D, Chikamoto A, Tsuji A, Yamamura K, *et al.* The diagnostic role  
425 of the neutrophil-to-lymphocyte ratio in predicting pancreatic ductal adenocarcinoma in  
426 patients with pancreatic diseases. *Int J Clin Oncol* **2016**;21(5):940-5 doi 10.1007/s10147-016-  
427 0975-z.
- 428 11. Guo W, Lu X, Liu Q, Zhang T, Li P, Qiao W, *et al.* Prognostic value of neutrophil-to-lymphocyte  
429 ratio and platelet-to-lymphocyte ratio for breast cancer patients: An updated meta-analysis of  
430 17079 individuals. *Cancer Med* **2019**;8(9):4135-48 doi 10.1002/cam4.2281.
- 431 12. Mei Z, Shi L, Wang B, Yang J, Xiao Z, Du P, *et al.* Prognostic role of pretreatment blood  
432 neutrophil-to-lymphocyte ratio in advanced cancer survivors: A systematic review and meta-  
433 analysis of 66 cohort studies. *Cancer treatment reviews* **2017**;58:1-13 doi  
434 10.1016/j.ctrv.2017.05.005.
- 435 13. Baron U, Werner J, Schildknecht K, Schulze JJ, Mulu A, Liebert UG, *et al.* Epigenetic immune  
436 cell counting in human blood samples for immunodiagnostics. *Sci Transl Med* **2018**;10(452) doi  
437 10.1126/scitranslmed.aan3508.
- 438 14. Le Cornet C, Schildknecht K, Rossello Chornet A, Fortner RT, Gonzalez Maldonado S, Katzke VA,  
439 *et al.* Circulating Immune Cell Composition and Cancer Risk: A Prospective Study Using  
440 Epigenetic Cell Count Measures. *Cancer Res* **2020**;80(9):1885-92 doi 10.1158/0008-5472.CAN-  
441 19-3178.
- 442 15. Riboli E, Hunt KJ, Slimani N, Ferrari P, Norat T, Fahey M, *et al.* European Prospective  
443 Investigation into Cancer and Nutrition (EPIC): study populations and data collection. *Public*  
444 *Health Nutr* **2002**;5(6B):1113-24 doi 10.1079/PHN2002394.

16. Caboux E, Lallemand C, Ferro G, Hemon B, Mendy M, Biessy C, *et al.* Sources of pre-analytical variations in yield of DNA extracted from blood samples: analysis of 50,000 DNA samples in EPIC. *PloS one* **2012**;7(7):e39821 doi 10.1371/journal.pone.0039821.
17. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, *et al.* DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *European journal of immunology* **2007**;37(9):2378-89 doi 10.1002/eji.200737594.
18. Sehouli J, Loddenkemper C, Cornu T, Schwachula T, Hoffmuller U, Grutzkau A, *et al.* Epigenetic quantification of tumor-infiltrating T-lymphocytes. *Epigenetics : official journal of the DNA Methylation Society* **2011**;6(2):236-46.
19. Singh A, Yamamoto M, Ruan J, Choi JY, Gauvreau GM, Olek S, *et al.* Th17/Treg ratio derived using DNA methylation analysis is associated with the late phase asthmatic response. *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology* **2014**;10(1):32 doi 10.1186/1710-1492-10-32.
20. Baron U, Turbachova I, Hellwag A, Eckhardt F, Berlin K, Hoffmuller U, *et al.* DNA methylation analysis as a tool for cell typing. *Epigenetics : official journal of the DNA Methylation Society* **2006**;1(1):55-60 doi 10.4161/epi.1.1.2643.
21. Schlittgen R. Analysis of incomplete multivariate data: J.L. Shafer (1997): Chapman & Hall, London, 430 pp., GB £ 39.00, ISBN 0-412-04061-1. *Computational Statistics & Data Analysis* **1999**;30(4):478-9 doi [https://doi.org/10.1016/S0167-9473\(99\)90025-7](https://doi.org/10.1016/S0167-9473(99)90025-7).
22. Sakaguchi S, Wing K, Yamaguchi T. Dynamics of peripheral tolerance and immune regulation mediated by Treg. *European journal of immunology* **2009**;39(9):2331-6 doi 10.1002/eji.200939688.
23. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* **2008**;133(5):775-87 doi 10.1016/j.cell.2008.05.009.
24. Orhan A, Vogelsang RP, Andersen MB, Madsen MT, Holmich ER, Raskov H, *et al.* The prognostic value of tumour-infiltrating lymphocytes in pancreatic cancer: a systematic review and meta-analysis. *Eur J Cancer* **2020**;132:71-84 doi 10.1016/j.ejca.2020.03.013.
25. Martinez-Bosch N, Vinaixa J, Navarro P. Immune Evasion in Pancreatic Cancer: From Mechanisms to Therapy. *Cancers (Basel)* **2018**;10(1) doi 10.3390/cancers10010006.
26. Schnell A, Schmidl C, Herr W, Siska PJ. The Peripheral and Intratumoral Immune Cell Landscape in Cancer Patients: A Proxy for Tumor Biology and a Tool for Outcome Prediction. *Biomedicines* **2018**;6(1) doi 10.3390/biomedicines6010025.
27. Turbachova I, Schwachula T, Vasconcelos I, Mustea A, Baldinger T, Jones KA, *et al.* The cellular ratio of immune tolerance (immunoCRIT) is a definite marker for aggressiveness of solid tumors and may explain tumor dissemination patterns. *Epigenetics : official journal of the DNA Methylation Society* **2013**;8(11):1226-35 doi 10.4161/epi.26334.
28. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* **2011**;331(6024):1565-70 doi 10.1126/science.1203486.
29. Maitra A, Hruban RH. Pancreatic cancer. *Annu Rev Pathol* **2008**;3:157-88 doi 10.1146/annurev.pathmechdis.3.121806.154305.
30. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. *The New England journal of medicine* **2014**;371(11):1039-49 doi 10.1056/NEJMra1404198.
31. Carnevale S, Ghasemi S, Rigatelli A, Jaillon S. The complexity of neutrophils in health and disease: Focus on cancer. *Semin Immunol* **2020**:101409 doi 10.1016/j.smim.2020.101409.
32. Liew PX, Kubes P. The Neutrophil's Role During Health and Disease. *Physiological reviews* **2019**;99(2):1223-48 doi 10.1152/physrev.00012.2018.
33. Tao L, Zhang L, Peng Y, Tao M, Li G, Xiu D, *et al.* Preoperative neutrophil-to-lymphocyte ratio and tumor-related factors to predict lymph node metastasis in patients with pancreatic ductal adenocarcinoma (PDAC). *Oncotarget* **2016**;7(45):74314-24 doi 10.18632/oncotarget.11031.
34. Ben Q, An W, Wang L, Wang W, Yu L, Yuan Y. Validation of the pretreatment neutrophil-lymphocyte ratio as a predictor of overall survival in a cohort of patients with pancreatic ductal adenocarcinoma. *Pancreas* **2015**;44(3):471-7 doi 10.1097/MPA.0000000000000271.



35. Cupp MA, Cariolou M, Tzoulaki I, Aune D, Evangelou E, Berlanga-Taylor AJ. Neutrophil to lymphocyte ratio and cancer prognosis: an umbrella review of systematic reviews and meta-analyses of observational studies. *BMC Med* **2020**;18(1):360 doi 10.1186/s12916-020-01817-1.
36. Yang JJ, Hu ZG, Shi WX, Deng T, He SQ, Yuan SG. Prognostic significance of neutrophil to lymphocyte ratio in pancreatic cancer: a meta-analysis. *World journal of gastroenterology : WJG* **2015**;21(9):2807-15 doi 10.3748/wjg.v21.i9.2807.
37. Michaud DS, Ruan M, Koestler DC, Alonso L, Molina-Montes E, Pei D, *et al.* DNA Methylation-Derived Immune Cell Profiles, CpG Markers of Inflammation, and Pancreatic Cancer Risk. *Cancer Epidemiol Biomarkers Prev* **2020**;29(8):1577-85 doi 10.1158/1055-9965.EPI-20-0378.

**Table 1: Baseline characteristics of the study subjects in the nested case-control study within EPIC [median (min–max) or n (%)]**

Variable	Cases (n=502)	Controls (n=502)
Women	248 (49)	248 (49)
Age at recruitment [years]	57 (30-76)	57 (30-76)
Age at diagnosis [years]	64 (37-87)	-
Follow-up [years]	8 (0-16)	
BMI [kg/m <sup>2</sup> ]		
<25	193 (38)	224 (45)
≥25	309 (62)	278 (55)
Alcohol intake at recruitment [g/d] <sup>1</sup>		
Men	15 (0-138)	14 (0-147)
Women	3 (0-81)	3 (0-59)
Smoking status <sup>1</sup>		
Never	187 (37)	222 (44)
Former	148 (30)	164 (33)
Current	162 (32)	114 (23)
Pack-years	5.0 (0-86)	0.4 (0-81)
Diabetes status <sup>1</sup>		
Self-reported diabetes at recruitment	29 (6)	19 (4)
No diabetes	418 (83)	434 (87)
Counts of immune markers relative to total nucleated cells [%] <sup>1,2</sup>		
Neutrophils	55 (3-107)	54 (4-97)
CD3+	22 (4-60)	22 (6-54)
CD8+	7 (1-28)	7 (0-27)
CD4+	15 (2-40)	15 (1-42)
FOXP3+	1 (0-4)	1 (0-4)
FOXP3-	14 (1-37)	14 (0-39)

<sup>1</sup>Missing (n case/n control): alcohol intake at recruitment (2/3), smoking status (5/2), pack year (72/55), diabetes (55/49), neutrophils (62/49), CD3+ (7/10), CD8+ (9/9), CD4+ (15/12), FOXP3+ (26/29), FOXP3- is the difference between CD4 and FOXP3+.

<sup>2</sup>Immune marker averages calculated after multiple imputation and re-calibration. Neutrophils were not re-calibrated; therefore individual percentage can exceed 100.

**Table 2.** Odds ratios (ORs) for the association between circulating immune cell composition (relative counts) and pancreatic cancer risk.

		Neutrophils	T cells					Model fit improvement p-value <sup>b</sup>
	Models <sup>a</sup>		Total CD3+	CD8+	CD4+	FOXP3+	FOXP3–	
All (502 cases / 502 controls)								
Cells modeled individually	M1	1.01 (1.00,1.02)	0.99 (0.97,1.01)					
Cells modeled with mutual adjustments across T-lymphocyte cells	M2			0.99 (0.95,1.03)	0.99 (0.96,1.02)			P <sub>M1M2</sub> =0.89
	M3			0.99 (0.95,1.03)		1.18 (0.89,1.57)	0.98 (0.94,1.01)	P <sub>M2M3</sub> =0.22 P <sub>M1M3</sub> =0.45
Lag Time < 5 years (147 cases / 147 controls)								
Cells modeled individually	M1	1.01 (0.99,1.03)	0.99 (0.96,1.03)					
Cells modeled with mutual adjustments across T-lymphocyte cells	M2			0.93 (0.86,1.01)	1.03 (0.98,1.08)			P <sub>M1M2</sub> =0.08
	M3			0.93 (0.85,1.00)		1.80 (1.01,3.23)	0.99 (0.94,1.06)	P <sub>M2M3</sub> =0.05 P <sub>M1M3</sub> =0.03
Lag Time 5 to 10 years (221 cases /221 controls)								
Cells modeled individually	M1	1.00 (0.99,1.02)	0.99 (0.96,1.02)					
Cells modeled with mutual adjustments across T-lymphocyte cells	M2			1.04 (0.98,1.10)	0.96 (0.92,1.00)			P <sub>M1M2</sub> =0.06
	M3			1.04 (0.98,1.10)		1.08 (0.70,1.66)	0.95 (0.90,1.00)	P <sub>M2M3</sub> =0.58 P <sub>M1M3</sub> =0.14
Lag Time 10 years or more (134 cases /134 controls)								
Cells modeled individually	M1	1.00 (0.98,1.03)	0.98 (0.95,1.02)					
Cells modeled with mutual adjustments across T-lymphocyte cells	M2			0.97 (0.90,1.04)	1.00 (0.93,1.07)			P <sub>M1M2</sub> =0.60
	M3			0.97 (0.90,1.05)		0.92 (0.53,1.62)	1.00 (0.93,1.08)	P <sub>M2M3</sub> =0.77 P <sub>M1M3</sub> =0.81

n=502 controls matched to 502 cases on study recruitment centre, sex, age at blood collection, date and time of blood collection, length of follow-up, and fasting status

<sup>a</sup>M1: model including CD3+ only, M2: model including CD8+ and CD4+, M3: model including CD8+, FOXP3+, FOXP3-.

<sup>b</sup>Improvement in fit between M1, M2, and M3.

Conditional logistic regression, modelling immune cells continuously as percent counts. Foxp3+ with unit 0.1

Fractions of CD4+ and CD8+ cells were re-calibrated so as to add up to the re-normalized fraction of total (CD3+) T cells; fractions of FOXP3+ and FOXP3- cells were re-calibrated so as to add up to fraction of re-normalized CD4+ cells.

**Figure 1.** Heatmap representing correlations between immune markers and selected baseline factors in 502 controls, adjusted for age and sex, and combined using Fisher's transformation

\* Denotes significance  $p < 0.05$

Missing (n case/n control): Alcohol average lifetime intake (87/82), waist circumference (56/56), Lifetime smoking in pack-year (72/55), time quitting smoking in former smokers (16/15), neutrophils (62/49), T-lymphocytes after normalization

