Plasma-derived CD16 exosomes and peripheral blood monocytes as correlating biomarkers in head and neck cancer

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Abstract. Exosomes play an important role in the individual immune regulation in patients with head and neck squamous cell carcinoma (HNSCC) as part of the tumor microenvironment. Patients with HNSCC with advanced tumor stages reveal significantly increased levels of plasma derived CD16^{+} (FcRIIIA) total exosomes as demonstrated in our previous study. Furthermore, increased individual abundances of peripheral blood CD16^{+} non-classical monocytes have been shown to correlate with increased monocytic programmed death ligand 1 (PD-L1) and CD4^{+} T cell disturbances in oropharyngeal cancer. However, the context of plasma derived CD16^{+} exosomes in patients with HNSCC and their role in the immune-regulation of circulating monocyte subsets has not been investigated so far. In the present study, exosomes were isolated from plasma samples of healthy donors and patients with HNSCC and evaluated for their morphology, size and protein composition using transmission electron microscopy, western blotting and bead-based flow cytometry. Monocyte subset abundances were analyzed in whole blood measurements in terms of the CD14/CD16 cell surface expression patterns, different monocytic adhesion molecules and checkpoint molecule PD-L1 using flow cytometry. Isolated exosomes were positive for the tetraspanins CD63 and CD9 as well as the endosomal marker TSG101, but negative for the non-exosomal marker glucose-regulated protein 94 and apolipoprotein ApoA1. Plasma derived CD16^{+} exosomes and exosome size distribution were significantly correlated with abundances of CD16^{+} non-classical monocytes and CD16^{+} intermediate monocytes, respectively. Furthermore, the data revealed significant correlations between CD16^{+} plasma derived exosomes and adhesion molecules CD29 (integrin β1) and CX3CR1 on certain monocyte subsets. These data suggested that CD16 positive exosomes and exosome size distribution were potential surrogates to characterize the composition of monocyte subsets in patients with HNSCC. Overall, both CD16-positive exosomes and CD16-positive monocyte subsets are potential liquid biomarkers that could be used to characterize the individual immunological situation of patients with HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common human cancer and develops from the mucosal epithelium in the oral cavity, pharynx, and larynx (1). Various cellular and molecular parameters influence individual immunological behaviors as well as different responses to therapeutic treatments in patients with head and neck cancer (2,3). In this context extracellular vesicles (EVs), especially exosomes, have gained increasing attention (4-6). Exosomes are small-sized (30-150 nm) EVs that are released by almost all types of cells and can be found in all body fluids. Tumor-derived exosomes play an important role for the intercellular communication and promote tumor progression and immune suppression (6,7).

We have recently shown that HNSCC patients with advanced tumor stages reveal significantly increased levels of plasma derived CD16^{+} total exosomes, suggesting CD16 positive exosomes as potential liquid biomarkers to characterize the individual situation of HNSCC patients (8). CD16 (FcRIIIA) is part of the Fc receptor (FcR) family and is known to be expressed on different immune cells such as natural killer (NK) cells, neutrophils and certain monocyte subsets (9,10). It has also been shown that cells of the human monocyte leukaemia cell line THP-1 (Tohoku Hospital Pediatrics-1) as well as THP-1 derived exosomes are strongly positive for CD16 (8).

Furthermore, our recent data revealed individual heterogeneous distributions of CD14/CD16 monocyte subset abundances in the peripheral blood of HNSCC patients compared to healthy donors, with elevated monocytic expression of checkpoint molecule PD-L1 in CD16^{+} non-classical

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monocytes in certain individuals (11). Briefly, peripheral blood monocytes can be subdivided based on their CD14 (a lipopolysaccharide co-receptor) and CD16 cell surface expression (10,12,13). CD14^{++}CD16$^-$ 'classical' monocytes have their origin in the bone marrow from where they enter the periphery. The so called 'intermediate' (CD14$^+$CD16$^+$) and 'non-classical' (CD14^{dim+}CD16$^+$) monocytes are considered as pro-inflammatory and more differentiated subtypes with specialized immune functions such as viral defense (14,15). Under normal conditions in healthy individuals, these subsets comprise each an amount of 5-10% of the entirety of peripheral blood monocytes. Increased abundances of these pro-inflammatory subsets have been identified in different acute and chronic inflammatory diseases (16-18). In cholangiocarcinoma as well as in colorectal cancer increased percentages of CD16$^+$ blood monocytes were associated with higher densities of tumor-associated macrophages (TAM) (19,20). Similarly, it has recently been observed that exosomes have an important impact on the communication between TAMs and cancer cells and therefore might be a promising target for innovative immunotherapeutic approaches (21).

However, the origin of plasma derived CD16$^+$ exosomes in HNSCC patients and their role within the immune-regulatory network of circulating monocytes has not been investigated so far. This study aimed to investigate the context of CD16$^+$ plasma-derived exosomes in terms of the differentiation patterns of peripheral blood CD14/CD16 monocyte subsets and the clinicopathological parameters in HNSCC patients. Furthermore, monocyte subsets were analyzed with regard to the expression patterns of adhesion molecules CD29 and CX3CR1 as well as immune checkpoint molecule programmed death ligand 1 (PD-L1) to broaden our understanding of the interplay of circulating monocyte subsets and exosomes as potential bioliquid mediators and indicators of tumor infiltration and progression.

Materials and methods

Ethics statement. All patients were subjected to standard clinical and surgical treatment between April and September 2021 at the Department of Otorhinolaryngology, University Hospital Schleswig-Holstein, Campus Luebeck, and have given their written informed consent for the following investigations. The study was approved by the local ethics committee of the University of Luebeck (approval number 16-278) and conducted in accordance with the ethical principles for medical research formulated in the WMA Declaration of Helsinki.

Exosome isolation. Exosomes were isolated from plasma samples of healthy volunteers and head and neck cancer patients by size exclusion chromatography as described previously (22). In short, freshly thawed plasma was sequentially centrifuged at 2,000 x g for 10 min and 10,000 x g for 30 min at 4°C. Following, the supernatant was filtered through 0.22 μm syringe-driven filters (Millipore, Burlington, MA, USA). 1 ml aliquots were loaded on pre-packed sepharose columns and eluted with PBS. Sequential 1 ml fraction #4 was collected and used for downstream analyses. Total exosome protein concentration was measured using Pierce BCA Protein Assay (Thermofisher Scientific, Waltham, MA, USA) according to

manufacturer's instructions. Exosomes were concentrated using 100 kDa cutoff centrifugal filters (Millipore).

Characterization of exosomes. Exosomes were characterized by western blot, nanoparticle tracking analysis and transmission electron microscopy. These methods are in line with the minimal information for studies of extracellular vesicle (MISEV) 2018 guidelines (23) and are routinely performed as described in our previous publication (8) (EV-TRACK ID: EV200068).

Bead-based flow cytometry of exosomes. Immune capture and bead-based flow cytometry were carried out as described previously (8,24,25). In short, 10 μg exosomes in 100 μl PBS were incubated with 1 μg biotin-labeled anti-CD63 (Cat: 353018, Biolegend, San Diego, CA, USA) for 2 h at room temperature on a shaker. Next, 10 μl ExoCap Streptavidin magnetic beads (MBL Life Science, Woburn, MA, USA) were added and incubated for another 2 h at room temperature on a shaker. Samples were washed using a magnetic rack and subsequently stained with the following antibodies/isotype controls for 1 h at room temperature on a shaker: CD14-PE (0.5 μg, Cat: 12-0149-42) and IgG1-PE (0.5 μg, Cat: 12-4714-42) (both from eBioscience/Thermofisher Scientific), CD16-APC (0.8 μg, Cat: 36076) and IgG1-APC (0.8 μg, Cat: 400122) (both from Biolegend), CD44v3-APC (10 μl, FAB5088A) and IgG2b-APC (10 μl, IC0041A) (both from R&D, Minneapolis, MN, USA). The stained complexes were washed twice using a magnetic rack and finally resuspended in 300 μl PBS for flow cytometry. Detection was performed using a Gallios flow cytometer with Kaluza 1.0 software (Beckman Coulter, Brea, CA, USA) and 10000 events were acquired. Data are presented as relative fluorescent intensity (RFI) which is the mean fluorescence intensity of the stained sample divided by the mean fluorescence intensity of the corresponding isotype control.

Blood collection and patient data. All blood donors have signed an informed written consent, and were clarified about the content of the proposed study. Blood (8 ml per patient) was drawn by venipuncture into a sodium citrate containing S-Monovette (Sarstedt; Nümbrecht, Germany). Blood samples were collected from healthy donors (n=10; 6 female/4 male; mean age of 59, range from 22 to 84) and head and neck cancer patients (n=25; 7 female/18 male; mean age of 64, range from 50 to 86). All analyzed HNSCC patients were treatment naïve. Blood samples were centrifuged at 1,000 x g for 10 min. Plasma specimens were stored in aliquots at -80°C. The clinicopathological characteristics of patients are listed in Table I.

FACS analysis of monocyte subsets. For FACS analyisis, 20 μl of citrate blood was diluted in 80 μl PBS within 4 h after blood collection. Staining of blood cells was performed with the following antibodies (diluted 1:50) for 25 min staining in the dark: CD45-PE (Cat: 368510), CD14-FITC (Cat: 367116), CD16-BV-510 (Cat: 302048), HLA-DR-APC-Cy7 (Cat: 307618), PD-L1-APC (Cat: 329708), CD29-PE-Cy7 (Cat: 303026) and CX3CR1-BV421 (Cat: 341620) (all from Biolegend, San Diego, USA). Afterward, 650 μl RBC Lysis Buffer (Biolegend) were added to the samples for another 20 min before the samples were centrifuged (400 x g for

Table I. Clinicopathological parameters.

Characteristics	Patients (n=25)	
	n	%
Sex		
Male	18	72
Female	7	28
Age (years)		
≤65	12	48
>65	13	52
Tumor site		
Pharynx	13	52
Larynx	5	20
Oral cavity	7	28
Tumor stage		
T1-t2	16	64
T3-t4	9	36
HPV status		
Positive	11	44
Negative	14	56
Alcohol abuse		
Yes	5	20
No	20	80
Tobacco consumption		
Yes	16	64
No	9	36

5 min) and supernatants were discarded. Cell pellets were resuspended in 100 μl fresh PBS and flow cytometry measurements were performed using a MACSQuant 10 flow cytometer (Miltenyi Biotec, Bergisch-Gladbach, Germany). Measured data were analyzed using the FlowJo software version 10.0 (FlowJo, LLC, Ashland, USA). All antibody titrations and compensations were performed beforehand. For whole blood measurements, at least 100 000 CD45$^+$ leukocytes were analyzed. Gating of monocyte subsets was performed as described before (26).

Immunohistochemistry and Evaluation. IHC staining was performed according to the manufacturer's instructions, using the Ventana Discovery (Ventana Medical System, Roche, Basel, Switzerland) automated staining system. In brief, slides were stained with the anti-PD-L1 antibody (rabbit monoclonal antibody, clone E1L3N, RTU; Cell Signaling, Danvers, MA, USA) and samples were evaluated by two independent pathologists. The tumor positivity score (TPS) was calculated as the percentage of tumor cells with positive PD-L1 membrane staining (range 0-100%).

Statistical analysis. GraphPad Prism Version 7.0f (GraphPad Software, Inc., San Diego, CA, USA) was used for unpaired student's t-Tests for statistical analysis of all data presented here. Analyses of patients samples were performed once per sample. The mean and standard errors (SEM) are presented.

$P<0.05$ was considered to indicate a statistically significant difference. Correlation analysis between different parameters was calculated using multivariate regression with the Pearson correlation coefficient. $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***).

Results

Characterization of exosomes from plasma of healthy donors and HNSCC patients. Exosomes isolated from plasma of healthy donors and HNSCC patients were evaluated for morphology, size and protein composition by transmission electron microscopy, nanoparticle tracking and western blot analysis. Isolated vesicles had a circular shape (Fig. 1A) and ranged from 30 to 200 nm with median diameters around 90 nm (Fig. 1B). Exosome preparations were positive for the tetraspanins CD63 and CD9 as well as the endosomal marker TSG101 but negative for the non-exosomal marker Grp94 and apolipoprotein ApoA1 (Fig. 1C). According to the MISEV 2018 guidelines (23), the described results allow for exosome nomenclature. Exosomal surface levels of CD44v3, used as tumor marker, were higher in HNSCC patients compared to healhty donors (HD) (Fig. 1D), indicating the presence of tumor-derived exosomes (TEX). Analysis of exosomal CD16 and CD14 surface values revealed no significant overall differences in HNSCC patients compared to healthy volunteers, but individual patients with increased levels of plasma derived CD16$^+$ exosomes (Fig. 2).

CD14/CD16 monocyte subsets of the HNSCC cohort. Circulating monocytes were divided into the three subsets CD14^{++}CD16$^-$ (classical), CD14^{++}CD16$^+$ (intermediate) and CD14^{dim+}CD16$^+$ (non-classical) using flow cytometry (27). Briefly, CD45 was used as a pan leukocyte marker for whole blood measurement and monocytes were first roughly gated by their FSC/SSC characteristics and the positivity for CD14 and CD16. Neutrophil granulocytes and NK-cells were excluded by their missing HLA-DR expression. Remaining B cells were excluded by the help of their lack of CD14 expression. Remaining monocytes were subgated into CD14^{++}CD16$^-$ (classical), CD14^{++}CD16$^+$ (intermediate) and CD14^{dim+}CD16$^+$ (non-classical) monocytes. HNSCC patients revealed similar median percentages of both classical and non-classical monocyte subsets compared to healthy donors, but stronger dispersions of individual distributions. Our measurements identified significantly increased percentages of intermediate monocytes in healthy donors and a drop of classical monocytes accompanied by an increase of non-classical monocytes in four HNSCC patients (Fig. 3). Further Pearson's correlation analyses between the pathological records of the intra-tumoral PD-L1 evaluation (tumor positivity score, TPS) and CD16 positive exosomes and monocytes revealed no significant correlations (Fig. S1).

Correlation of CD16$^+$ exosomes and monocyte subset alterations. Our data revealed heterogeneous abundances of both plasma derived CD16$^+$ exosomes and circulating monocyte subsets in the analysed HNSCC patients. Correlation analysis between these parameters revealed a significant positive correlation (P=0.0312) between CD16$^+$ exosomes and CD16$^+$
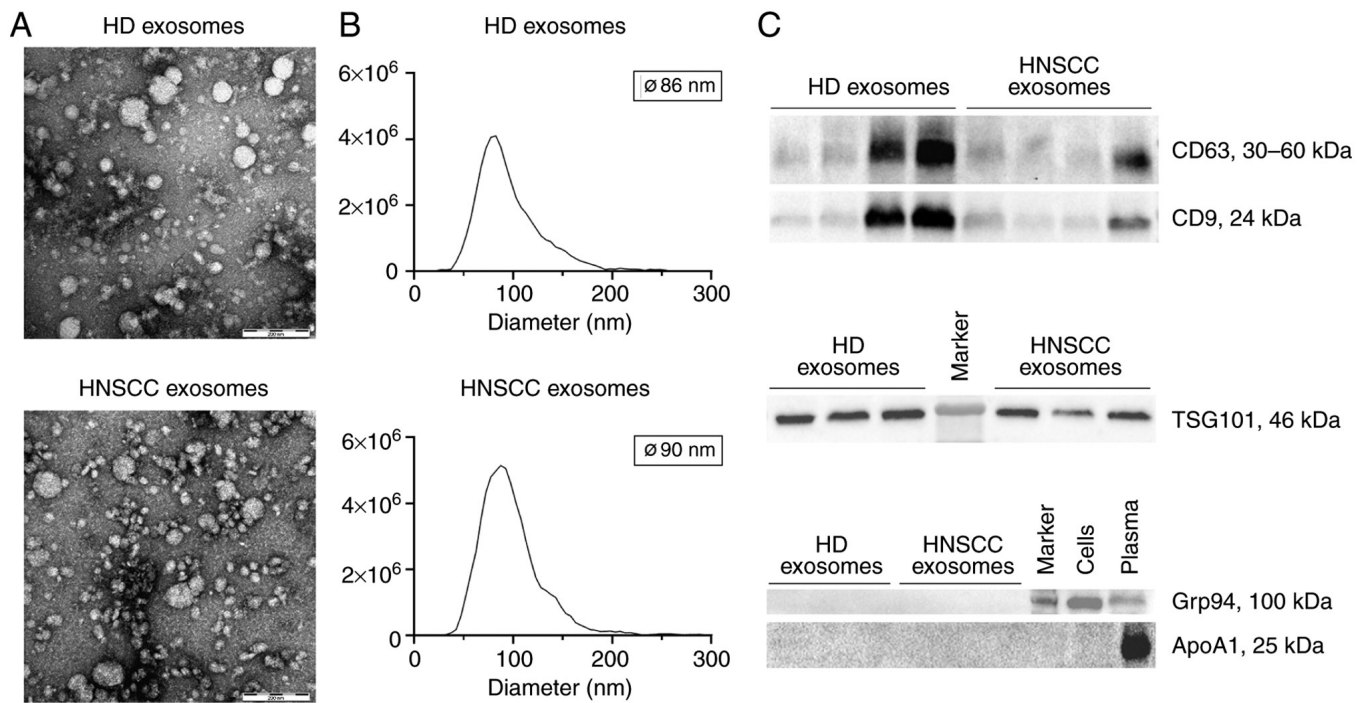
Figure 1. Characterization of exosomes from plasma of healthy donors and patients with HNSCC. (A) Representative transmission electron microscopy images of HD and HNSCC exosomes (scale bar, 200 nm). (B) Representative size distribution of HD and HNSCC exosomes as measured by nanoparticle tracking analysis. (C) Western blotting of HD and HNSCC exosomes for the presence of tetraspanins CD9 and CD63 and the endosomal marker TSG101 as well as the absence of cellular Grp94 and contaminant ApoA1. HNSCC cell lysate and plasma were used as positive control for Grp94 and ApoA1, respectively. HD, healthy donor; HNSCC, head and neck squamous cell carcinoma; Grp94, glucose-regulated protein 94; ApoA1, apolipoprotein A1; RFI, relative fluorescence intensity.

non-classical monocytes but not CD16+ intermediate monocytes in head and neck cancer patients (Fig. 4A). Additional correlation analysis between exosomal CD14, revealed no significant correlation (Fig. 4B). In contrast, correlation analyisis of both plasma derived CD14+ and CD16+ exosomes and circulating monocyte subsets in the analyzed healthy donors revealed no significant correlations (Fig. 5). Analysis of CD16+ exosomes from HPV positive and HPV negative patients revealed no significant differences (Fig. S2).

In addition, correlation analyses between exosomal CD16 percentages and CD16 expression of natural killer (NK) cells and neutrophils were performed. Our data reveleaed no significant correlation, which corroborates the conection between circulating CD16+ monocytes and plasma CD16 exosomes in HNSCC patients (Fig. 6). Furthermore, expression of adhesion molecules CD29 (integrin β1) and CX3CR1 was measured in all three monocyte subsets and correlated with the abundances of plasma derived CD16+ exosomes. Our data revealed significant positive correlations between CD16 plasma derived exosomes and CD29 expression on classical (P=0.0338) and non-classical monocytes (P=0.0474) monocytes as well as a significant correlation between CX3CR1 and CD16 plasma derived exosomes expression on intermediate monocytes (P=0.0192) (Fig. 7).

Of note, the four patients with the strongest monocyte subset alterations all suffer from oropharyngeal cancer, thus located in a lymphoid-tissue-rich anatomical region that may have a systemic influence on the immune regulation in the peripheral blood.

Next, the PD-L1 expression of monocytes was analyzed and correlated with exosome CD16 expression, exosome diameter values (nm) and total exome protein (μg/ml). Our data indicated significantly increased monocytic PD-L1 expression in HNSCC patients compared to healthy donors (P=0.0014) (Fig. 8A). Correlation analysis revealed no significant correlations between monocytic PD-L1 expression and exosomal CD16 and total exosomal protein levels, but a significant correlation between monocytic PD-L1 and exosome diameter values (Fig. 8). Further correlation analysis revealed a positive significant correlation between exosome diameter (nm) and percentages of classical monocytes as well as a significant negative correlation between exosome diameter (nm) and percentages of intermediate monocytes (Fig. 9).

Discussion

The present study was undertaken to investigate the possible connection between plasma derived CD16 exosomes and abundances and characteristics of circulating CD14/CD16 monocyte subsets in head and neck cancer. In general, the investigations revealed no significant overall differences of exosomal CD16 and CD14 values between HNSCC patients and healthy donors, but certain individuals with increased abundances of CD16+ exosomes. Monocyte subsets were distinguished using flow cytometry and revealed overall heterogeneous distributions in HNSCC patients and significantly increased percentages of intermediate monocytes in healthy donors. However, there are conflicting observations concerning the shift of certain monocyte subsets in head and neck cancer in the literature so far. In a recent study, investigations on a cohort of different HNSCC subtypes found lower abundances of intermediate and higher percentages of
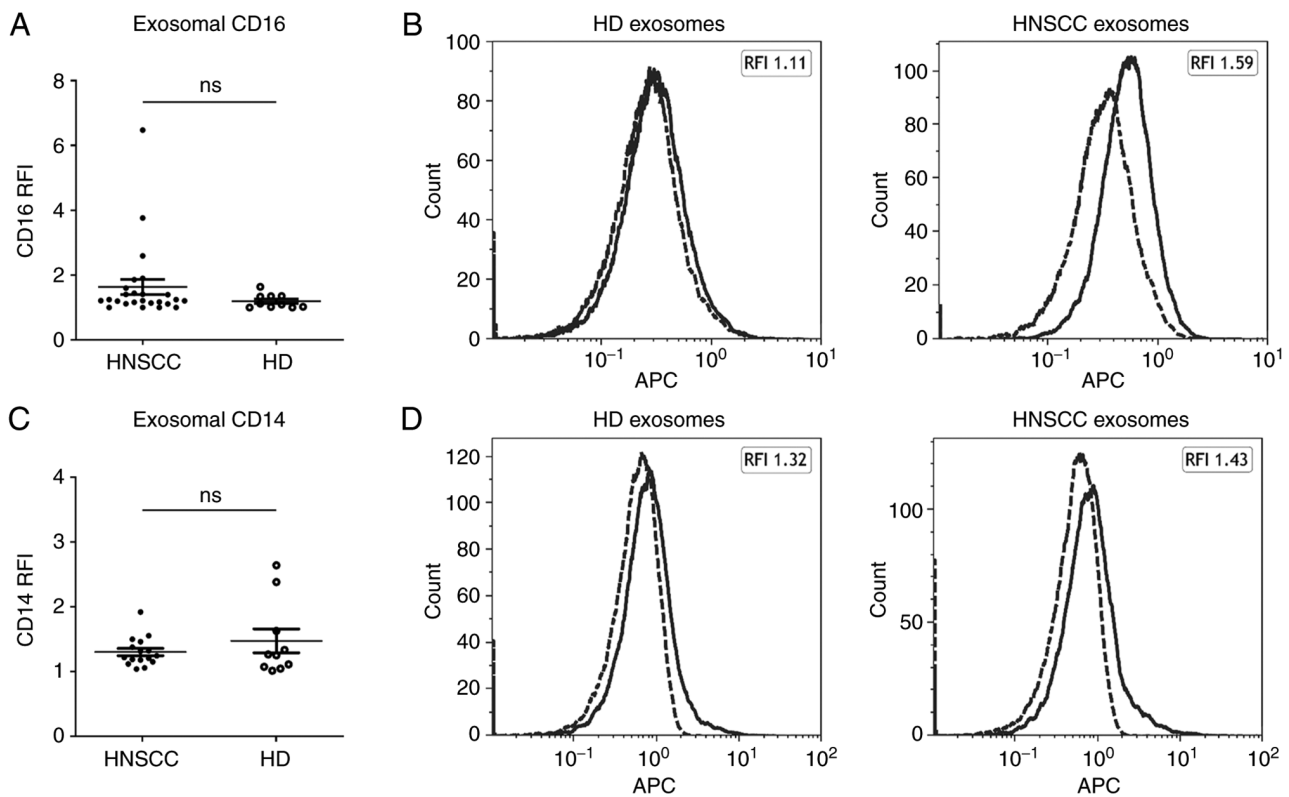
Figure 2. Exosomal cargo of HD and patients with HNSCC. Comparison of (A and B) exosomal CD16 and (C and D) exosomal CD14 values between HD and patients with HNSCC revealed no significant differences, although individual patients with HNSCC showed elevated exosomal CD16 values. Exosomal CD16 and CD14 values were determined using bead-based flow cytometry of CD63-captured exosomes. Results are shown as relative fluorescence intensity compared with an isotype control from patients with HNSCC. (A) and (C) present the mean and standard deviation. In the representative flow cytometry histograms depicting (B) CD16 (n=10 HD; n=25) and (D) CD14 (n=10 HD; n=16) values of HD and HNSCC exosomes, the solid line represents the CD16 or CD14 signal and the dashed line represents the isotype control. ns, not significant; HD, healthy donor; HNSCC, head and neck squamous cell carcinoma.
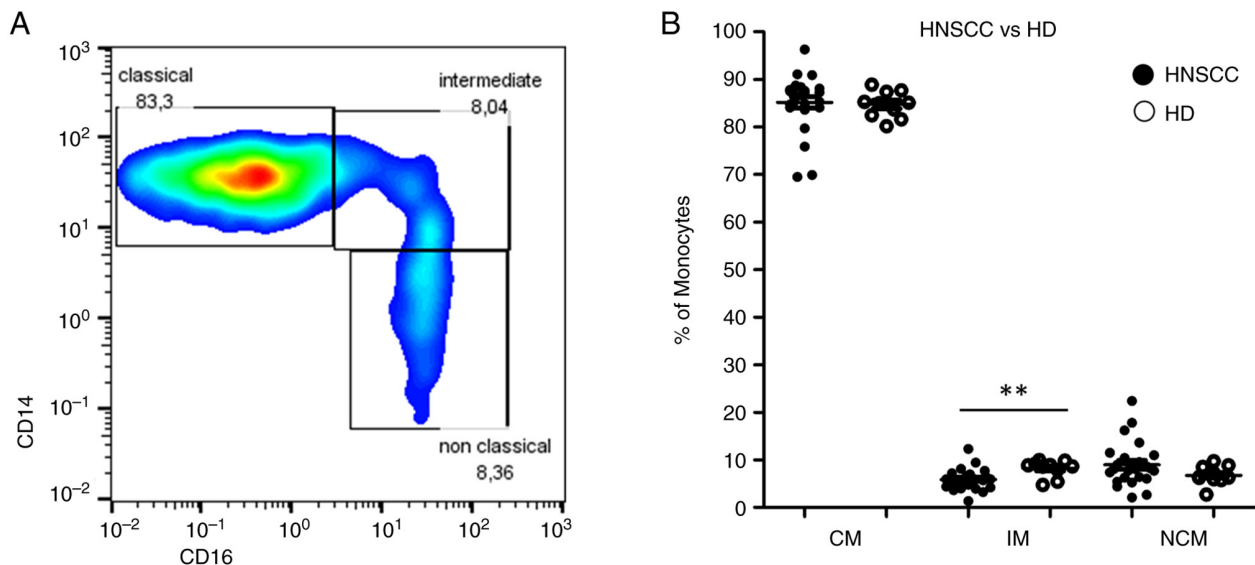

Figure 3. Analysis of CD14- and CD16-characterized monocyte subsets. (A) Representative gating pattern of CD14/CD16 monocyte subset analysis by flow cytometry. (B) Whole blood measurements revealed similar median abundances of classical monocytes and non-classical monocytes in patients with HNSCC (n=25) compared with healthy donors (n=10), but a larger spreading of monocyte subset distributions in patients with cancer. Patients with HNSCC revealed significantly decreased percentages of IM. **$P<0.01$. CM, classical monocytes; NCM, non-classical monocytes; IM, intermediate monocytes; HD, healthy donor; HNSCC, head and neck squamous cell carcinoma.

classical monocytes in comparison to healthy donors (28). Another cohort of 22 oropharyngeal cancer patients revealed

increased percentages of classical monocytes and no differences of non-classical monocytes compared to healthy
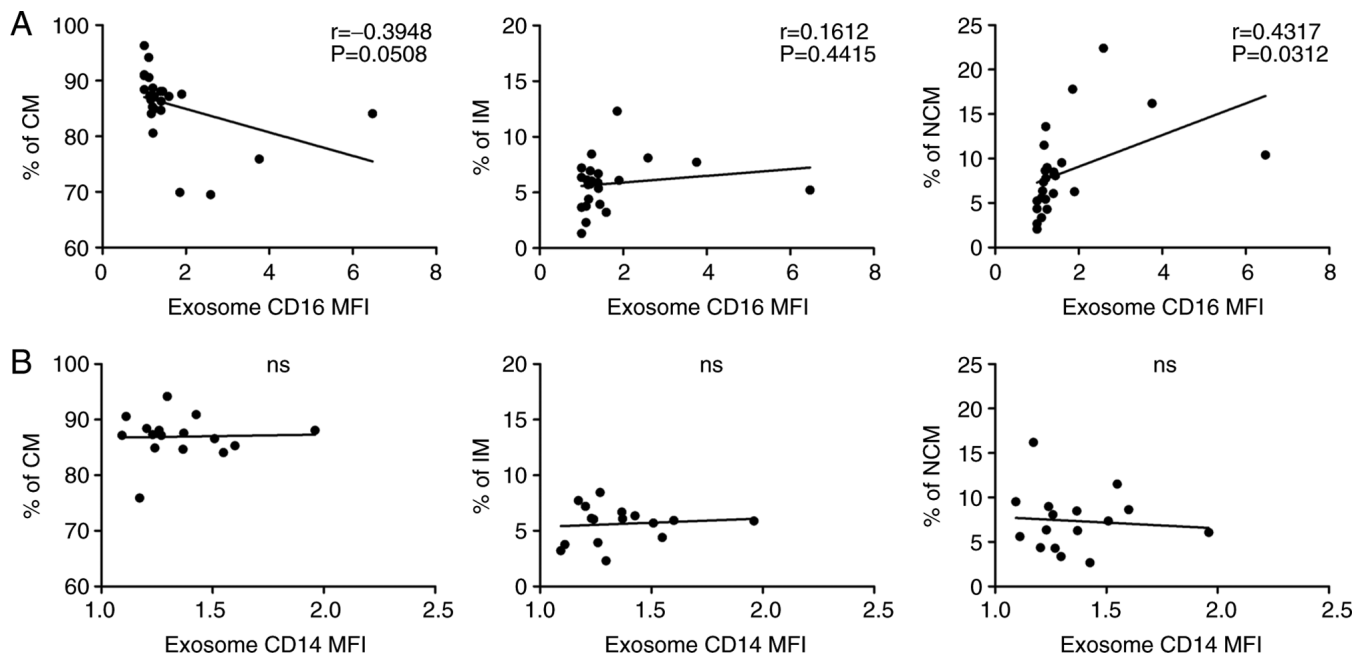
Figure 4. Correlations between exosomes and monocyte subsets in HNSCC. Correlation analysis between exosomal (A) CD16 and (B) CD14 and percentages (of total monocytes) of CM, IM and NCM in patients with HNSCC. Data revealed a significant positive correlation between exosomal CD16 and percentages of NCM. The correlation coefficient (r) and P-values are given for each monocyte subset. P<0.05 was considered as significant. MFI, mean fluorescence intensity; CM, classical monocytes; NCM, non-classical monocytes; IM, intermediate monocytes; HNSCC, head and neck squamous cell carcinoma.
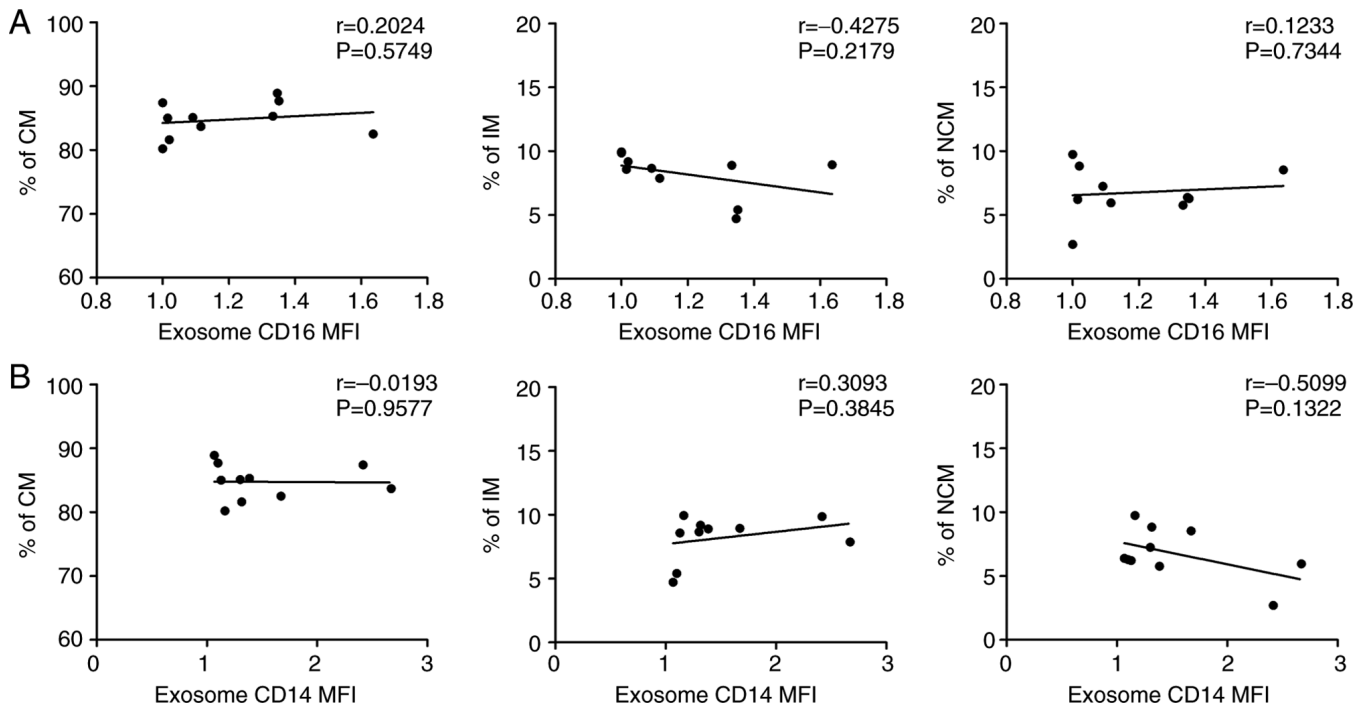

Figure 5. Correlations between exosomes and monocyte subsets in healthy donors. Correlation analysis between exosomal (A) CD16 and (B) CD14 and percentages (of total monocytes) of CM, IM and NCM in healthy donors. Data revealed no significant correlations. The correlation coefficient (r) and P-values are given for each monocyte subset. P<0.05 was considered as significant. MFI, mean fluorescence intensity; CM, classical monocytes; NCM, non-classical monocytes; IM, intermediate monocytes.

donors (29). These different observations are most likely due to the different sizes of the analyzed patient cohorts and the manifold individual parameters in each cohort that influence the monocyte subset distribution. This is as well an acknowledged limitation of the present study. We found a significant positive correlation between plasma derived CD16 exosomes and circulating CD16^{+} non-classical monocytes, suggesting these cells as potential origin of CD16 plasma derived exosomes in head and neck cancer. It is most likely that the induction of CD16^{+} monocytes in certain individuals is tumor
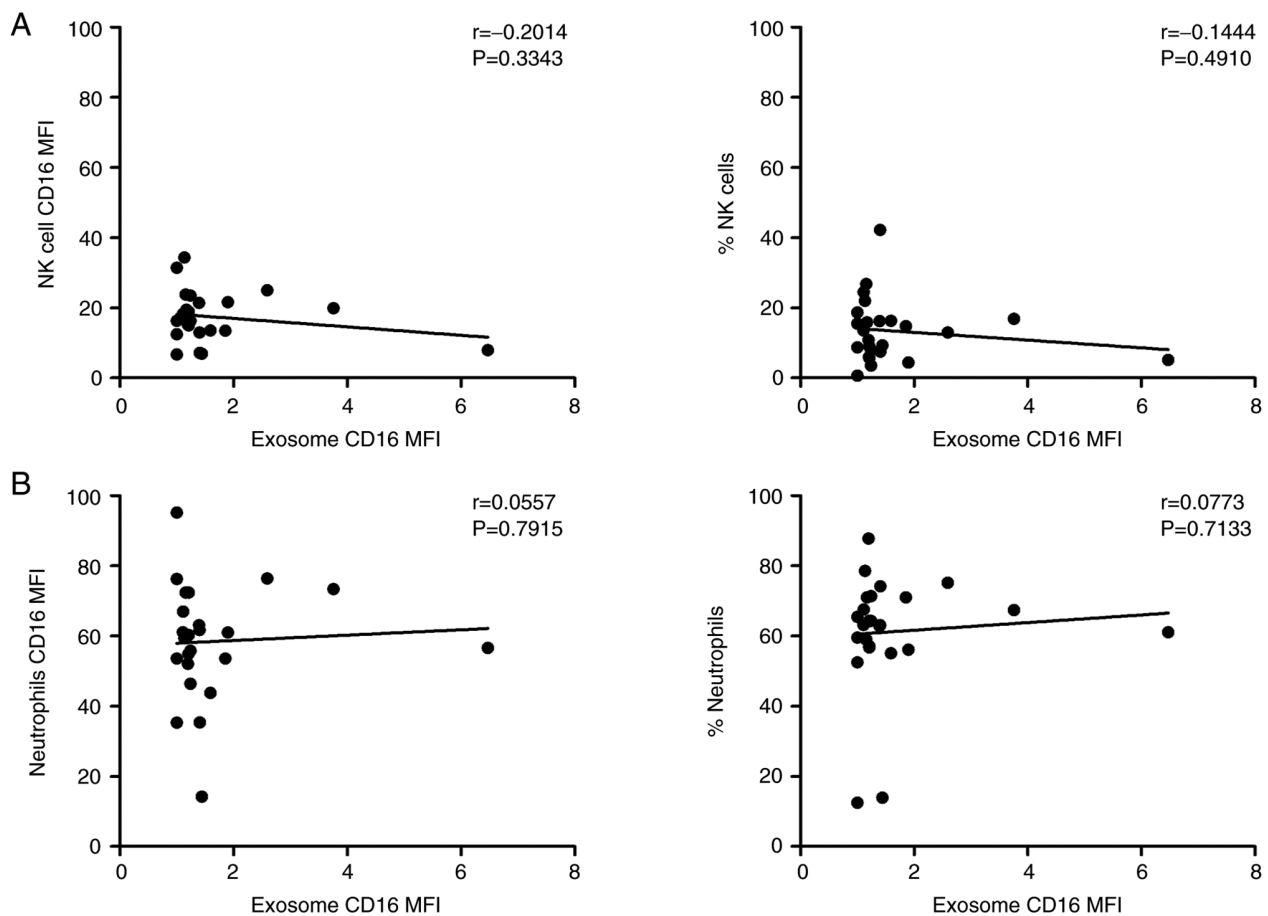
Figure 6. Correlations between exosomes, NK cells and neutrophils. Correlation analysis between exosomal CD16 and percentages and CD16 expression of (A) NK cells and (B) neutrophils. Data revealed no significant correlations. The correlation coefficient (r) and P-values are given for each monocyte subset. P<0.05 was considered as significant. MFI, mean fluorescence intensity; NK, natural killer.

driven, since a reconstitution of monocyte subset distribution was observed after tumor resection in patients with cholangiocarcinoma (30). Besides their correlation with clinical parameters, plasma exosomes have as well been identified as a promising bioliquid indicator for treatment response and tumor progression in head and neck cancer (31).

In this study, total exosomes from plasma of HNSCC patients and HD were analyzed and they represent a mixture of exosomes derived from normal, tumor and immune cells. We confirmed the presence of CD44v3+ TEX in HNSCC patients. Previous studies showed that HNSCC patients have higher amounts of total exosomes compared to HD and that these are mainly tumor- and immune cell-derived (22,24,25,32). Remarkably, CD63 and CD9 showed interindivudal variability while TSG101 was the most stable exosomal marker. This variance of exosomal markers is commonly observed between different tumor cell lines (33) and patients (32,34). For exosome nomenclature, however, the presence of both transmembrane proteins (CD9, CD63) and cytosolic proteins (TSG101) recovered in exosomes need to be analyzed, irrespective of the exact composition.

Fc receptor CD16 (FcRIIIA) is known to be expressed as well on natural killer (NK) cells and neutrophils. We can't distinguish from which cell populations CD16 exosomes derive but studies suggest reduced NK cell numbers expressing CD16 (35,36) and downregulated CD16 on NK

cells (37,38) in HNSCC, rendering it less likely that CD16 NK cells contribute to CD16 exosomes to a large extent. Correspondingly, our correlation analyses between exosomal CD16 percentages and CD16 expression of natural killer (NK) cells and neutrophils revealed no significant correlation, which corroborates the connection between circulating CD16+ monocytes and plasma-derived CD16 exosomes in HNSCC patients. Our data revealed significant correlations between CD16 plasma derived exosomes and CD29 (integrin β1) expression on classical and non-classical monocytes. β1- and β2-integrins play an important role in the regulation of cell attachment and migration of immune cells in inflammatory processes (39). These data may indicate an impact of plasma derived exosomes on the adhesion characteristics and intra-tumoral migration of circulating monocytes. It has recently been shown in colorectal cancer, that tumor cell-derived spondin 2 triggers the transendothelial migration of monocytes via the integrin β1/PYK2 axis. Furthermore, inhibition of the integrin β1/PYK2 axis was found to impair the transendothelial migration of monocytes as well as the cancer-promoting functions of tumor-associated macrophages (40). Furthermore, we identified a significant correlation between CD16+ plasma derived exosomes and CX3CR1 expression on intermediate monocytes. Adhesion molecule CX3CR1 is required for monocyte crawling along the blood vessels by mediating the interaction
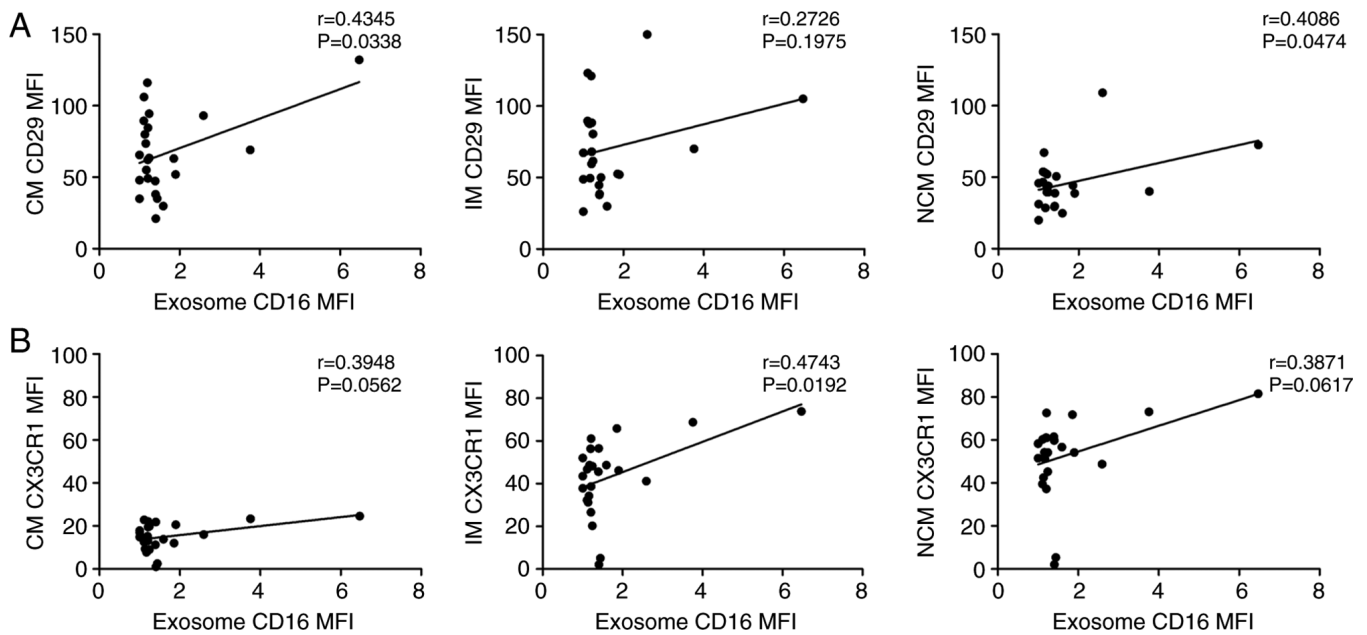
Figure 7. CD14/CD16 exosomes and monocyte subsets. Correlation analysis between exosomal (A) CD16 and (B) CD14 and percentages of CM, IM and NCM in patients with HNSCC. Data revealed a significant positive correlation between exosomal CD16 and percentages of NCM. The correlation coefficient (r) and P-values are given for each monocyte subset. P<0.05 was considered as significant. MFI, mean fluorescence intensity; CM, classical monocytes; NCM, non-classical monocytes; IM, intermediate monocytes; HNSCC, head and neck squamous cell carcinoma.
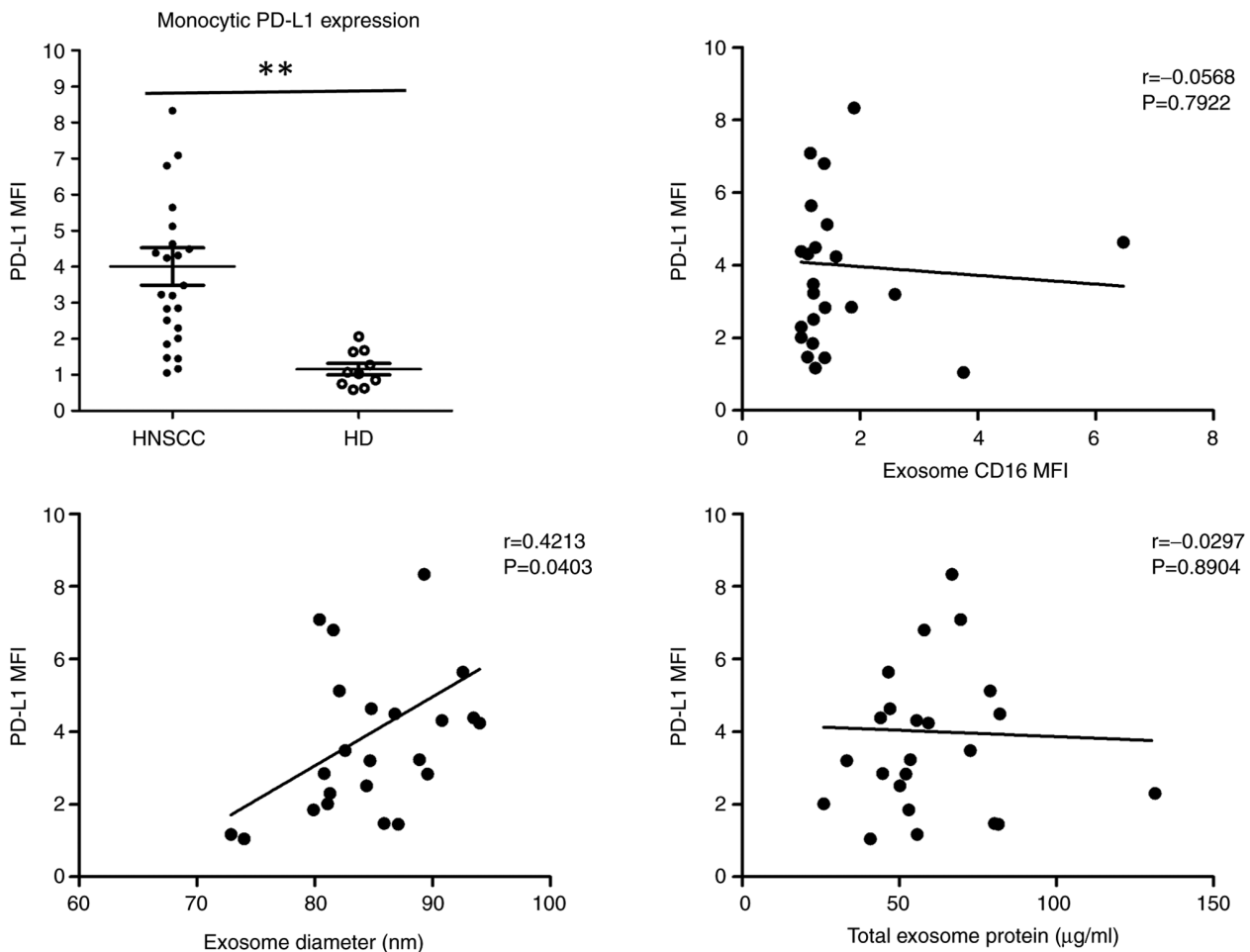

Figure 8. Correlation analysis between monocytic PD-L1 expression and exosome values. (A) Flow cytometric measurements show significantly increased monocytic PD-L1 expression in patients with HNSCC compared with healthy donors. Correlation analysis between monocytic PD-L1 expression and (B) exosome CD16 expression, (C) exosome diameter and (D) total exosome protein. The correlation coefficient (r) and P-values are given for each correlation. P<0.05 was considered as significant. MFI, mean fluorescence intensity; PD-L1, programmed death ligand 1; HNSCC, head and neck squamous cell carcinoma.
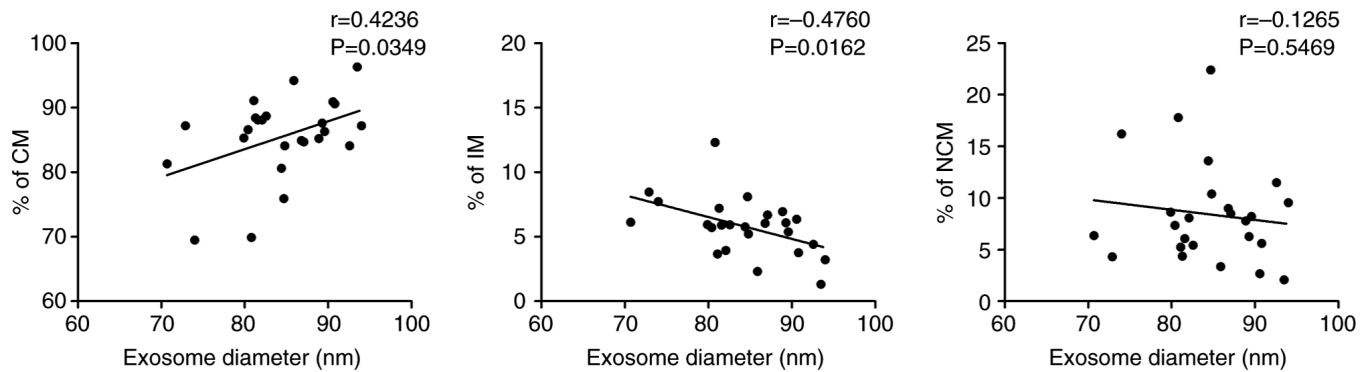
Figure 9. Correlation analysis between exosome diameter (nm) and percentages of monocyte subsets (of total monocytes) from patients with HNSCC. The correlation coefficient (r) and P-values are given for each correlation. P<0.05 was considered as significant.

with the endothelium (41). Correspondingly, CX3CR1 is as well associated with atherosclerosis and vascular inflammatory processes (42,43). In patients with skin cancer, CX3CR1 has been shown to contribute to the accumulation of tumor-associated macrophages (44).

Measurements revealed significantly increased monocytic PD-L1 expression in HNSCC patients compared to healthy patients, which corroborates earlier studies (11). Checkpoint molecule PD-L1 plays an important role in tumor immune escape mechanisms by inhibiting T-cell responses via its counterpart programmed death 1 (PD1) (45). Further correlation analysis revealed a significant correlation between monocytic PD-L1 expression and measured exosome diameter values, maybe due to increased protein cargo of certain exosome subsets.

Surprisingly, further correlation analysis revealed a significant positive correlation between exosome diameter (nm) and percentages of classical monocytes as well as a significant negative correlation between exosome diameter (nm) and percentages of intermediate monocytes. This means that increased abundances of intermediate monocytes accompany with a reduced size of plasma exosomes in HNSCC patients.

It has recently been shown in prostate cancer (PC) patients that plasmatic exosomes were smaller in size compared to healthy individuals (46). It has been proposed that the altered characteristics of plasmatic exosomes in PC patients may be driven by tumor microenvironmental conditions, such as acidity (47) as well as the tumor mass (48,49).

However, our data suggest that characteristics of plasma exosomes and immune alterations of circulating monocytes in HNSCC patients are directly related or at least accompany upon tumor driven alterations of the individual microenvironment. The establishment of individual bioliquid markers for diagnosis and therapy of cancer patients requires the long term comprehensive analysis of countless molecular and cellular parameters in correlation with the clinical situation. There will not be the 'one biomarker' with clinical relevance for all patients but rather a complex interaction of various potential biomarkers for each individual cancer patient. Thus, the present work is just a small but novel and necessary contribution to the big picture. This is the first study to show that plasma-derived exosomes can inform us about the state of monocyte subsets in corresponding blood samples in HNSCC patients. Not only a correlation with non-canonical monocytes was visible, but also with adhesion

molecules and checkpoint molecule PD-L1, which enable immune cells to migrate and to regulate immune functions.

An acknowledged limitation of the present study is the relatively small size of the analyzed patient cohort. Further investigations on larger patient cohorts are required to understand the biological induction as well as the clinical consequences of the exosome mediated inter-monocytic communication in HNSCC patients. Furthermore, patient outcome over a longer period of time should be observed to assess the potential usability of the analyzed parameters as bioliquid markers for patient prognosis and therapy response prediction.

We do not propose the direct use of exosome CD16 for the treatment of HNSCC, but we focus on its use as marker for the level of immune suppression. By understanding the immune status of patients, appropriate therapies can be selected. Our data suggest that not only tumor derived but also monocyte derived exosomes participate in the communication and immune alteration within the circulating monocyte subpopulations in head and neck cancer. Plasma-derived exosomes have the potential to reflect the immune status of the patient with the long-term aim of easily characterizing the TME as high or low immune suppressive and facilitating the selection of the most suitable therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LH, DH, CI, JF, MNT and RP carried out the molecular studies, data curation and statistical analysis. LH, CB, TKH,

KLB, MNT and RP participated in the design and coordination of the study and helped to draft the manuscript. MNT and RP confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients were treated surgically at the Department of Otorhinolaryngology, University Hospital Schleswig-Holstein, Campus Luebeck, and have given their written informed consent for the following investigations. The study was approved by the local ethics committee of the University of Luebeck (approval no. 16-278) and conducted in accordance with the ethical principles for medical research formulated in the WMA Declaration of Helsinki. Informed consent was obtained from all subjects involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Zhang P, Li S, Zhang T, Cui F, Shi JH, Zhao F and Sheng X: Characterization of molecular subtypes in head and neck squamous cell carcinoma with distinct prognosis and treatment responsiveness. Front Cell Dev Biol 9: 711348, 2021.
2. Alsahafi E, Begg K, Amelio I, Raulf N, Lucarelli P, Sauter T and Tavassoli M: Clinical update on head and neck cancer: Molecular biology and ongoing challenges. Cell Death Dis 10: 540, 2019.
3. Canning M, Guo G, Yu M, Myint C, Groves MW, Byrd JK and Cui Y: Heterogeneity of the head and neck squamous cell carcinoma immune landscape and its impact on immunotherapy. Front Cell Dev Biol 7: 52, 2019.
4. Whiteside TL: The effect of tumor-derived exosomes on immune regulation and cancer immunotherapy. Future Oncol 13: 2583-2592, 2017.
5. Whiteside TL: Exosomes carrying immunoinhibitory proteins and their role in cancer. Clin Exp Immunol 189: 259-267, 2017.
6. Whiteside TL: Exosomes in cancer: Another mechanism of tumor-induced immune suppression. Adv Exp Med Biol 1036: 81-89, 2017.
7. Milane L, Singh A, Mattheolabakis G, Suresh M and Amiji MM: Exosome mediated communication within the tumor microenvironment. J Control Release 219: 278-294, 2015.
8. Hofmann L, Ludwig S, Schuler PJ, Hoffmann TK, Brunner C and Theodoraki MN: The potential of CD16 on plasma-derived exosomes as a liquid biomarker in head and neck cancer. Int J Mol Sci 21: 3739, 2020.
9. Barros-Martins J, Bruni E, Fichtner AS, Cornberg M and Prinz I: OMIP-084: 28-Color full spectrum flow cytometry panel for the comprehensive analysis of human γδ T cells. Cytometry A 101: 856-861, 2022.
10. Ziegler-Heitbrock L: Blood monocytes and their subsets: Established features and open questions. Front Immunol 6: 423, 2015.
11. Idel C, Loyal K, Rades D, Hakim SG, Schumacher U, Bruchhage KL and Pries R: Smoking-, alcohol-, and age-related alterations of blood monocyte subsets and circulating CD4/CD8 T cells in head and neck cancer. Biology (Basel) 11: 658, 2022.
12. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM and Wong SC: The three human monocyte subsets: Implications for health and disease. Immunol Res 53: 41-57, 2012.
13. Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA, Bigley V, Flavell RA, Gilroy DW, Asquith B, *et al*: The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. J Exp Med 214: 1913-1923, 2017.
14. Boyette LB, Macedo C, Hadi K, Elinoff BD, Walters JT, Ramaswami B, Chalasani G, Taboas JM, Lakkis FG and Metes DM: Phenotype, function, and differentiation potential of human monocyte subsets. PLoS One 12: e0176460, 2017.
15. Jakubzick CV, Randolph GJ and Henson PM: Monocyte differentiation and antigen-presenting functions. Nat Rev Immunol 17: 349-362, 2017.
16. Rossol M, Kraus S, Pierer M, Baerwald C and Wagner U: The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. Arthritis Rheum 64: 671-677, 2012.
17. Moniuszko M, Bodzenta-Lukaszyk A, Kowal K, Lenczewska D and Dabrowska M: Enhanced frequencies of CD14++CD16+, but not CD14+CD16+, peripheral blood monocytes in severe asthmatic patients. Clin Immunol 130: 338-346, 2009.
18. Azeredo EL, Neves-Souza PC, Alvarenga AR, Reis SR, Torrentes-Carvalho A, Zagne SM, Nogueira RM, Oliveira-Pinto LM and Kubelka CF: Differential regulation of toll-like receptor-2, toll-like receptor-4, CD16 and human leucocyte antigen-DR on peripheral blood monocytes during mild and severe dengue fever. Immunology 130: 202-216, 2010.
19. Subimerb C, Pinlaor S, Khuntikeo N, Leelayuwat C, Morris A, McGrath MS and Wongkham S: Tissue invasive macrophage density is correlated with prognosis in cholangiocarcinoma. Mol Med Rep 3: 597-605, 2010.
20. Schauer D, Starlinger P, Reiter C, Jahn N, Zajc P, Buchberger E, Bachleitner-Hofmann T, Bergmann M, Stift A, Gruenberger T and Brostjan C: Intermediate monocytes but not TIE2-expressing monocytes are a sensitive diagnostic indicator for colorectal cancer. PLoS One 7: e44450, 2012.
21. Jiang H, Zhou L, Shen N, Ning X, Wu D, Jiang K and Huang X: M1 macrophage-derived exosomes and their key molecule lncRNA HOTTIP suppress head and neck squamous cell carcinoma progression by upregulating the TLR5/NF-κB pathway. Cell Death Dis 13: 183, 2022.
22. Hong CS, Funk S, Muller L, Boyiadzis M and Whiteside TL: Isolation of biologically active and morphologically intact exosomes from plasma of patients with cancer. J Extracell Vesicles 5: 29289, 2016.
23. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, *et al*: Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 7: 1535750, 2018.
24. Theodoraki MN, Matsumoto A, Beccard I, Hoffmann TK and Whiteside TL: CD44v3 protein-carrying tumor-derived exosomes in HNSCC patients' plasma as potential noninvasive biomarkers of disease activity. Oncoimmunology 9: 1747732, 2020.
25. Theodoraki MN, Hoffmann TK and Whiteside TL: Separation of plasma-derived exosomes into CD3(+) and CD3(-) fractions allows for association of immune cell and tumour cell markers with disease activity in HNSCC patients. Clin Exp Immunol 192: 271-283, 2018.
26. Polasky C, Steffen A, Loyal K, Lange C, Bruchhage KL and Pries R: Reconstitution of monocyte subsets and PD-L1 expression but Not T cell PD-1 expression in obstructive sleep apnea patients upon PAP therapy. Int J Mol Sci 22: 11375, 2021.
27. Polasky C, Steffen A, Loyal K, Lange C, Bruchhage KL and Pries R: Redistribution of monocyte subsets in obstructive sleep apnea syndrome patients leads to an imbalanced PD-1/PD-L1 cross-talk with CD4/CD8 T cells. J Immunol 206: 51-58, 2021.
28. Sakakura K, Takahashi H, Motegi SI, Yokobori-Kuwabara Y, Oyama T and Chikamatsu K: Immunological features of circulating monocyte subsets in patients with squamous cell carcinoma of the head and neck. Clin Immunol 225: 108677, 2021.
29. Takahashi H, Sakakura K, Tada H, Kaira K, Oyama T and Chikamatsu K: Prognostic significance and population dynamics of peripheral monocytes in patients with oropharyngeal squamous cell carcinoma. Head Neck 41: 1880-1888, 2019.
30. Subimerb C, Pinlaor S, Lulitanond V, Khuntikeo N, Okada S, McGrath MS and Wongkham S: Circulating CD14(+) CD16(+) monocyte levels predict tissue invasive character of cholangiocarcinoma. Clin Exp Immunol 161: 471-479, 2010.
31. Theodoraki MN, Laban S, Jackson EK, Lotfi R, Schuler PJ, Brunner C, Hoffmann TK, Whiteside TL and Hofmann L: Changes in circulating exosome molecular profiles following surgery/(chemo)radiotherapy: Early detection of response in head and neck cancer patients. Br J Cancer 125: 1677-1686, 2021.

32. Ludwig S, Floros T, Theodoraki MN, Hong CS, Jackson EK, Lang S and Whiteside TL: Suppression of lymphocyte functions by plasma exosomes correlates with disease activity in patients with head and neck cancer. Clin Cancer Res 23: 4843-4854, 2017.
33. Yoshioka Y, Konishi Y, Kosaka N, Katsuda T, Kato T and Ochiya T: Comparative marker analysis of extracellular vesicles in different human cancer types. J Extracell Vesicles 2, 2013.
34. Hofmann L, Abou Kors T, Ezić J, Niesler B, Röth R, Ludwig S, Laban S, Schuler PJ, Hoffmann TK, Brunner C, et al: Comparison of plasma- and saliva-derived exosomal miRNA profiles reveals diagnostic potential in head and neck cancer. Front Cell Dev Biol 10: 971596, 2022.
35. Bose A, Chakraborty T, Chakraborty K, Pal S and Baral R: Dysregulation in immune functions is reflected in tumor cell cytotoxicity by peripheral blood mononuclear cells from head and neck squamous cell carcinoma patients. Cancer Immun 8: 10, 2008.
36. Türkseven MR and Oygür T: Evaluation of natural killer cell defense in oral squamous cell carcinoma. Oral Oncol 46: e34-e37, 2010.
37. Watanabe M, Kono K, Kawaguchi Y, Mizukami Y, Mimura K, Maruyama T, Izawa S and Fujii H: NK cell dysfunction with down-regulated CD16 and up-regulated CD56 molecules in patients with esophageal squamous cell carcinoma. Dis Esophagus 23: 675-681, 2010.
38. Dasgupta S, Bhattacharya-Chatterjee M, O'Malley BW Jr and Chatterjee SK: Inhibition of NK cell activity through TGF-beta 1 by down-regulation of NKG2D in a murine model of head and neck cancer. J Immunol 175: 5541-5550, 2005.
39. Ou Z, Dolmatova E, Lassègue B and Griendling KK: β1- and β2-integrins: Central players in regulating vascular permeability and leukocyte recruitment during acute inflammation. Am J Physiol Heart Circ Physiol 320: H734-H739, 2021.
40. Huang C, Ou R, Chen X, Zhang Y, Li J, Liang Y, Zhu X, Liu L, Li M, Lin D, et al: Tumor cell-derived SPON2 promotes M2-polarized tumor-associated macrophage infiltration and cancer progression by activating PYK2 in CRC. J Exp Clin Cancer Res 40: 304, 2021.
41. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G and Geissmann F: Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 317: 666-670, 2007.
42. McDermott DH, Halcox JP, Schenke WH, Waclawiw MA, Merrell MN, Epstein N, Quyyumi AA and Murphy PM: Association between polymorphism in the chemokine receptor CX3CR1 and coronary vascular endothelial dysfunction and atherosclerosis. Circ Res 89: 401-407, 2001.
43. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, Garin A, Liu J, Mack M, van Rooijen N, et al: Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. J Clin Invest 117: 185-194, 2007.
44. Ishida Y, Kuninaka Y, Yamamoto Y, Nosaka M, Kimura A, Furukawa F, Mukaida N and Kondo T: Pivotal involvement of the CX3CL1-CX3CR1 axis for the recruitment of M2 tumor-associated macrophages in skin carcinogenesis. J Invest Dermatol 140: 1951-1961.e6, 2020.
45. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, et al: Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192: 1027-1034, 2000.
46. Logozzi M, Mizzoni D, Di Raimo R, Giuliani A, Maggi M, Sciarra A and Fais S: Plasmatic exosome number and size distinguish prostate cancer patients from healthy individuals: A prospective clinical study. Front Oncol 11: 727317, 2021.
47. Logozzi M, Mizzoni D, Angelini DF, Di Raimo R, Falchi M, Battistini L and Fais S: Microenvironmental pH and exosome levels interplay in human cancer cell lines of different histotypes. Cancers (Basel) 10: 370, 2018.
48. Logozzi M, De Milito A, Lugini L, Borghi M, Calabrò L, Spada M, Perdicchio M, Marino ML, Federici C, Iessi E, et al: High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. PLoS One 4: e5219, 2009.
49. Rodríguez Zorrilla S, Pérez-Sayans M, Fais S, Logozzi M, Gallas Torreira M and García García A: A pilot clinical study on the prognostic relevance of plasmatic exosomes levels in oral squamous cell carcinoma patients. Cancers (Basel) 11: 429, 2019.