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RESEARCH ARTICLE

Tumor Markers and Signatures

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Prognostic significance of circulating tumor cells and tumor related transcripts in small cell lung cancer: A step further to clinical implementation

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Abstract

Small-cell lung cancer (SCLC) is a fatal disease with limited treatment options. Circulating tumor cells (CTCs) in liquid biopsy samples may serve as predictive and prognostic biomarkers; but the analysis of CTCs is still challenging. By using microfluidic or density gradient CTC enrichment in combination with immunofluorescent (IF) staining or qPCR of CTC-related transcripts, we achieved a 60.8% to 88.0% positivity in SCLC blood samples. Epithelial and neuroendocrine transcripts including the druggable target *DLL3* were associated with shorter overall survival (OS), indicating the clinical value of these markers in terms of differential diagnosis and treatment decisions. High CTC counts and the presence of CTC duplets detected by IF staining were prognostic for OS, and thus may serve as indicators of disease progression or therapy failure. In patient samples with high CTC load detected by IF staining, a concordance of the transcripts positivity in circulating free plasma RNA and CTCs was observed. Our data emphasize the role of CTCs and CTC-related transcripts and underline the clinical value of liquid biopsy analysis in SCLC.

KEYWORDS

circulating free RNA, prognostic marker, circulating tumor cells, gene expression analysis, microfluidics

What's new?

Circulating tumor cells (CTCs) are detectable in most small cell lung cancers (SCLCs), often in relatively high numbers. But while CTCs are promising prognostic biomarkers in SCLC, further study is needed to optimize methods for their detection. Here, the authors investigated the

Abbreviations: CDKN1B, cyclin-dependent kinase inhibitor 1B; cfRNA, circulating free plasma RNA; CHGA, chromogranin A; CK19, cytokeratin 19; CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; DAPI, 4′,6-diamidino-2-phenylindol; DLL3, delta-like-protein 3; EpCAM, epithelial cell adhesion molecule 1; FDA, US Food and Drug Administration; HD, healthy donor; IF, immunofluorescent; LC, Lightcycler; NSCLC, non-small cell lung cancer; OQ, OncoQuick; OS, overall survival; PABAK, kappa test adjusted for low prevalence and bias; PBMCs, peripheral blood mononuclear cells; PD-L1, programmed cell death 1 ligand 1; PX, Parsortix; qPCR, quantitative polymerase chain reaction; RT, room temperature; SCLC, small cell lung cancer; SYP, synaptophysin; WBC, white blood cell.

utility of liquid biopsy analysis of CTCs for SCLC using a combination of approaches for CTC enrichment and immunofluorescent staining or qPCR of CTC-related transcripts. Pre-amplification for CTC-related transcript detection using qPCR maximized the number of positive findings in SCLC. Detection of cancer-related gene transcripts and high CTC counts were linked to shorter overall survival among SCLC patients.

1 | INTRODUCTION

Lung cancer, the second most common cancer affecting both sexes was estimated in 2.2 million new cases worldwide (2020), accounting for 11.4% of all new cancer diagnoses and for 18.0% of all cancer related deaths.[1–3] In the last decade, the treatment of lung cancer has substantially changed with the advent of targeted therapies and immunotherapies for patients with non-small cell lung cancer (NSCLC), which constitutes the major histopathological subtype of the disease in comparison to small cell lung cancer (SCLC). The management of NSCLC patients has been tremendously revolutionized by the discovery of actionable oncogenic driver mutations and the introduction of individualized targeted therapy. Liquid biopsy samples (mostly peripheral blood) can complement tissue biopsies to identify targetable mutations in cell-free circulating tumor DNA (ctDNA) isolated from the blood plasma. The analysis of ctDNA can aid in selecting patients for first-line treatment; but it is also clinically relevant in the second-line setting, when liquid biopsies taken at serial time points enable the detection of emergent resistance mutations in real-time.[4,5]

Unlike with NSCLC, there are no targeted therapy options available for patients diagnosed with SCLC, which accounts for 15% of all lung cancer cases. SCLC is the most aggressive lung cancer subtype; it grows rapidly and tends to disseminate early. Due to the aggressiveness of the disease, the prognosis of SCLC is poor, with a median survival of less than a year for patients with extensive disease at first diagnosis.[6] The standard first-line therapy is platinum-based chemotherapy in combination with immunotherapy; response to treatment is usually assessed by imaging diagnostics.

SCLC has neuroendocrine features and in 85% of the SCLC cells the notch ligand delta-like ligand 3 (DLL3) is overexpressed. Due to its low or even absent expression in non-cancerous cells, DLL3 serves as diagnostic marker and has been evaluated as potential therapeutic drug target.[7] SCLC patients rarely undergo surgery and thus tumor tissue samples are often limited for therapy decision making. As SCLC patients have a relatively higher number of disseminated circulating tumor cells (CTCs) in their blood as compared to NSCLC patients,[8] CTCs may serve as surrogate of tumor tissue for prognostic and predictive information, for monitoring the course of the disease, and studying mechanisms of resistance. Due to the limited advancements in effective treatment options or targeted agents, an important role of liquid biopsies in SCLC may be the generation of patient derived explants from CTCs for preclinical research.[9]

The analysis of CTCs is usually preceded by an enrichment or isolation step to increase the relative abundance of these rare cells in the sample. The plethora of available technologies are generally based on the distinct functional or physical properties of the CTCs as compared to the surrounding blood cells.[10] For example, the CellSearch system (Menarini) captures epithelial CTCs expressing the EpCAM protein (epithelial cell adhesion molecule 1) on the cell surface using specific antibodies bound to magnetic nanoparticles. Similarly, immunocapture of CTCs expressing EpCAM and/or other surface markers is also employed by other technologies such as the AdnaTests (Qiagen). Furthermore, miscellaneous label-free methods have been developed to isolate CTCs by filtration (e.g., ISET, Rarecells), density gradient centrifugation (e.g., OncoQuick, Greiner Bio-One), microfluidic separation (e.g., Parsortix, Angle plc.), dielectrophoretic field-flow fractionation (ApoStream, ApoCell) and by applying hydrodynamic forces. The reason for using label-free methods is the heterogeneity of CTCs and the possible downregulation or even lack of EpCAM expression. Once isolated, CTCs are then further analyzed by DNA, RNA, or protein based techniques allowing for enumeration and molecular characterization of the cells.

Several studies have already shown the prognostic value of CTCs in SCLC, most of them using the FDA (US Food and Drug Administration) cleared CellSearch test,[11–16] which identifies and enumerates EpCAM-positive CTCs by immunofluorescent staining (IF) of intracellular cytokeratins. Furthermore, label-free technologies such as the Parsortix (PX) microfluidic system were used to enable epitope-independent enrichment of CTCs, resulting in a higher number of CTC-positive samples compared to epitope-dependent enrichment.[17,18] In our previous study we established a workflow for the molecular detection of epithelial- and neuroendocrine-specific gene transcripts (including the emerging drug target DLL3[7]) by quantitative PCR (qPCR) in SCLC blood samples enriched by the PX system.[19] The detection of CTCs based on their gene expression can represent an interesting alternative to conventional IF staining, as qPCR is characterized by extreme sensitivity and further allow the analysis of multiple transcripts of interest in a single sample.

However, in view of the generally high number of CTCs in SCLC, the number of positive patient samples was lower than expected in our study.[19] Thus, we hereby aimed to improve the CTC analysis strategy to increase the overall positivity. For this purpose, we optimized our previously established workflow combining CTC enrichment using PX and molecular characterization of the enriched cells by gene expression analysis. Similar to our previous study, we analyzed epithelial- (*EpCAM*, *CK19*) and neuroendocrine-specific (*SYP*, *CHGA* and *DLL3)* gene transcripts. We evaluated a target-specific pre-amplification of CTC-related transcripts prior to qPCR and a less stringent enrichment procedure, reducing the depletion of peripheral blood mononuclear cells (PBMCs) and increasing the recovery of

CTCs. CTC enumeration by rare cell detection based on immunofluorescent staining using the novel nCyteDx Platform, was also evaluated in this study. The label-free nCyteDx technology isolates all PBMCs and potentially present CTCs via density gradient centrifugation. The isolated cells are then stained with the nPac proprietary immunofluorescent cocktail, scanned and analyzed for CTCs by an advanced cell finding and analysis software. The technology may have notable advantages over CellSearch, including the label-free enrichment and superior image quality.[20]

In addition to the investigation of CTCs, we assessed circulating free plasma RNA (cfRNA), which has gained research interest in the past few years, as potential biomarker for liquid biopsy analysis in SCLC. cfRNA is transported in the blood via vesicles or bound to proteins and is thereby stabilized.[21] While ctDNA may reflect the tumor burden, cfRNA can be a marker of tumor evolution.[22] Based on a recent study pointing to the potential clinical applicability of cfRNA in lung cancer,[23] we investigated for the first time the same CTC-related gene expression markers (*EpCAM*, *CK19*, *SYP*, *CHGA* and *DLL3*) in cfRNA isolated from SCLC plasma samples.

2 | MATERIALS AND METHODS

2.1 | Patients and blood samples

Blood samples were drawn aseptically from lung cancer patients at the Department of Respiratory and Critical Care Medicine, Klinik Floridsdorf, Vienna, Austria and samples for control purpose were obtained from healthy donors (HD) scheduled for platelet apheresis at the Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Vienna, Austria. The SCLC subtype was diagnosed by histologic examination of a specimen. The clinical characteristics of the patients are given in Tables S3 and S4. To avoid contamination, the first few milliliters were discarded and subsequently 23 to 30 ml blood was collected in four Vacuette EDTA tubes (Greiner Bio-One) for molecular analyses, and approximately 9 ml were drawn in CellSave preservative tube (Menarini Silicon Biosystems Inc.) for IF staining.

2.2 | Blood processing for subsequent molecular analyses

Peripheral blood samples taken in Vacuette EDTA tubes were processed with a microfluidic PX cassette with a critical step size of 6.5 µm (GEN3D6.5) at 99 mbar pressure. The captured cells were harvested and immediately lysed. In order to evaluate less stringent enrichment procedures, the blood sample was split into two equal parts and processed with a PX cassette having a critical step size of just 4.5 µm (GEN3D4.5) and with OncoQuick (OQ) density gradient centrifugation (Greiner Bio-One). After OQ separation, the top plasma layer containing cfRNA was centrifuged at 3000×*g* for 15 min at room temperature (RT). The enriched cells at the interphase between plasma and density gradient medium were washed and immediately lysed.

2.3 | RNA isolation

Total RNA was isolated without DNase treatment using the RNeasy Micro Kit (Qiagen) from the cell lysates according to manufacturer's instructions.

Yeast carrier tRNA (Sigma) and a spike-in template[24] were added to previously prepared 3 ml plasma. cfRNA isolation was then carried out using the QIAamp ccfDNA/RNA kit (Qiagen).

2.4 | Reverse transcription, pre-amplification and qPCR

Half of the total RNA and cfRNA was reverse transcribed using the SuperScript VILO Mastermix (Invitrogen). The targets of interest (*SYP*, *CHGA*, *EpCAM*, *CK19* and *DLL3*) as well as the reference gene *CDKN1B* were quantified as previously described[19] with or without a preceding target-specific pre-amplification of 10 cycles. For each marker, a sample was assigned as positive according to previously defined cut-off thresholds.[25] With the other half of the RNA samples *CK19* gene expression using hybridization probes and primers specific for the *CK19* gene[26] was analyzed on the Lightcycler 480 II (LC) (Roche) according to Welsch et al.[25] Due to the high specificity of the assay, any amplified transcript was considered positive.

2.5 | CTC detection by immunofluorescent staining

Six milliliter blood were transferred from the CellSave tube into a CPT Tube (BD Biosciences) and the PBMC fraction was isolated by centrifugation. CTCs were identified using the Circulating Epithelial Cell nPAC RUO Kit (Axon Dx, LLC) employing anti-panCK, anti-CK19, anti-CD45 and other proprietary white blood cell marker (WBC) according to the manufacturer's instruction. All stained cells were immobilized onto filter membranes (max. 1 million cells per membrane), which were transferred onto glass slides and scanned with the nCyte Dx platform (Axon Dx, LLC). Image acquisition and evaluation was performed with the nCyte Dx nAble software (Axon Dx, LLC). A CK+, DAPI+ and WBC- event with morphological features consistent with that of a nucleated cell was classified as a CTC.

2.6 | Statistics

McNemar test was used to compare the differences in transcript positivity rates between the respective protocols. To evaluate the level of concordance between the two enrichment methods (PX vs OQ), the Kappa test adjusted for low prevalence and bias (PABAK) was used.[27]

The associations of marker positivity and patients' characteristics were evaluated using a Fisher's exact test or a chi-square test. Kaplan-Meier survival analyses and log-rank testing were used to compare survival outcomes.[28] Overall survival (OS) was defined as the period of time in months between blood draw and either death or the last date the patient was seen alive. Two-sided tests were used at all analyses. IBM SPSS Statistics 21 was used for statistical analysis. Inkscape 1.2 was used for evaluation and representation of immunofluorescent images. R (version: 4.3.2) was used for graphics design and analysis.

3 | RESULTS

3.1 | CTC-related gene transcripts with and without pre-amplification

In the first part of the study, we asked whether a target-specific pre-amplification prior to the detection of CTC-related transcripts by qPCR would increase the number of positive findings. To answer this question, we processed 74 blood samples from 66 SCLC patients and 19 HD using the microfluidic GEN3D6.5 PX separation cassette, and then split the obtained RNA samples to compare qPCR with and without pre-amplification.

By qPCR without pre-amplification, 12/74 patient samples (16.2%) were assigned as positive due to the presence of at least one of the investigated transcripts. In contrast, the percentage of positive samples significantly increased (McNemar test; $p < 0.001$) by 3.8-fold due to the pre-amplification step, yielding in 45/74 (60.8%) positive samples. Each marker was significantly more often detected after pre-amplification (Table S1).

However, the introduction of a pre-amplification step also increased the background gene expression in HD samples with all markers except *CHGA* and made the introduction of a cut-off threshold value inevitable. Nevertheless, even after considering that threshold, 1/19 (5.3%) of HD samples was classified as *EpCAM* positive (Figure 1). When a minimum of two positive markers was defined as threshold for overall positivity of a sample, 8/74 (10.8%) patient samples without pre-amplification and 30/74 (40.5%) of samples with pre-amplification were classified as positive, whereas none of the HD samples.

Previously, we categorized the used markers into neuroendocrine (*CHGA* and *SYP*), and epithelial (*EPCAM* and *CK19*).[19] In the present

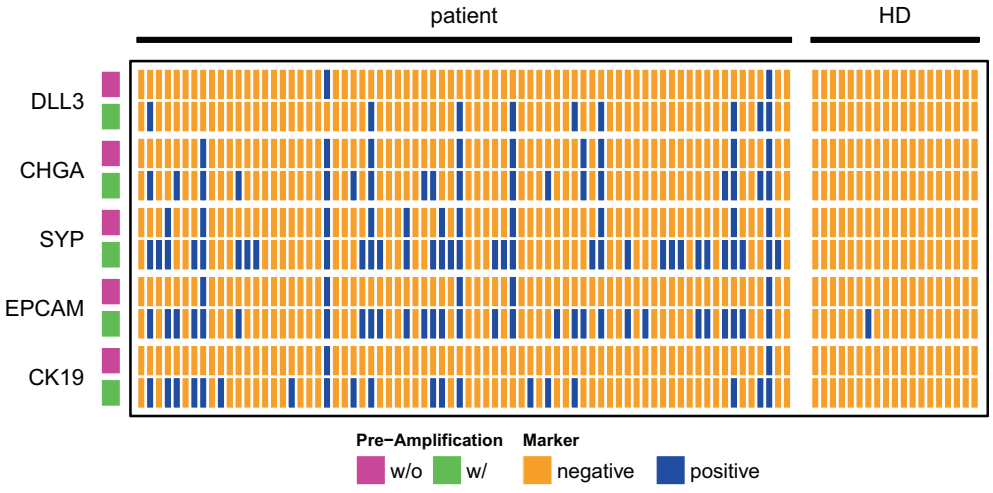

FIGURE 1 Heatmap showing SCLC patient (n = 74) and healthy donor (HD, n = 19) samples positivity of each marker by qPCR with and without pre-amplification after Parsortix CTC enrichment. For the definition of positivity, a threshold was set considering the gene expression levels in the HD group, in order to allow a maximum of 10% positive findings in the HD group. Samples with positive findings are marked blue and with negative findings orange.

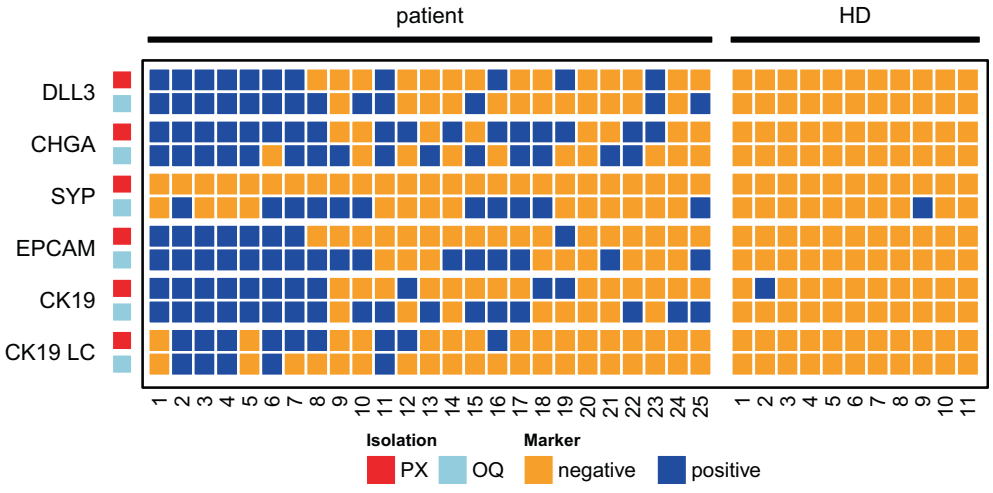

FIGURE 2 Heatmap showing SCLC patient (n = 25) and healthy donor (HD, n = 11) samples positivity of each marker after Parsortix (PX) or OncoQuick (OQ) CTC enrichment and qPCR analysis. For the definition of positivity, a threshold was set considering the gene expression levels in the HD group, in order to allow a maximum of 10% positive findings in the HD group. Samples with positive findings are marked blue and with negative findings orange.

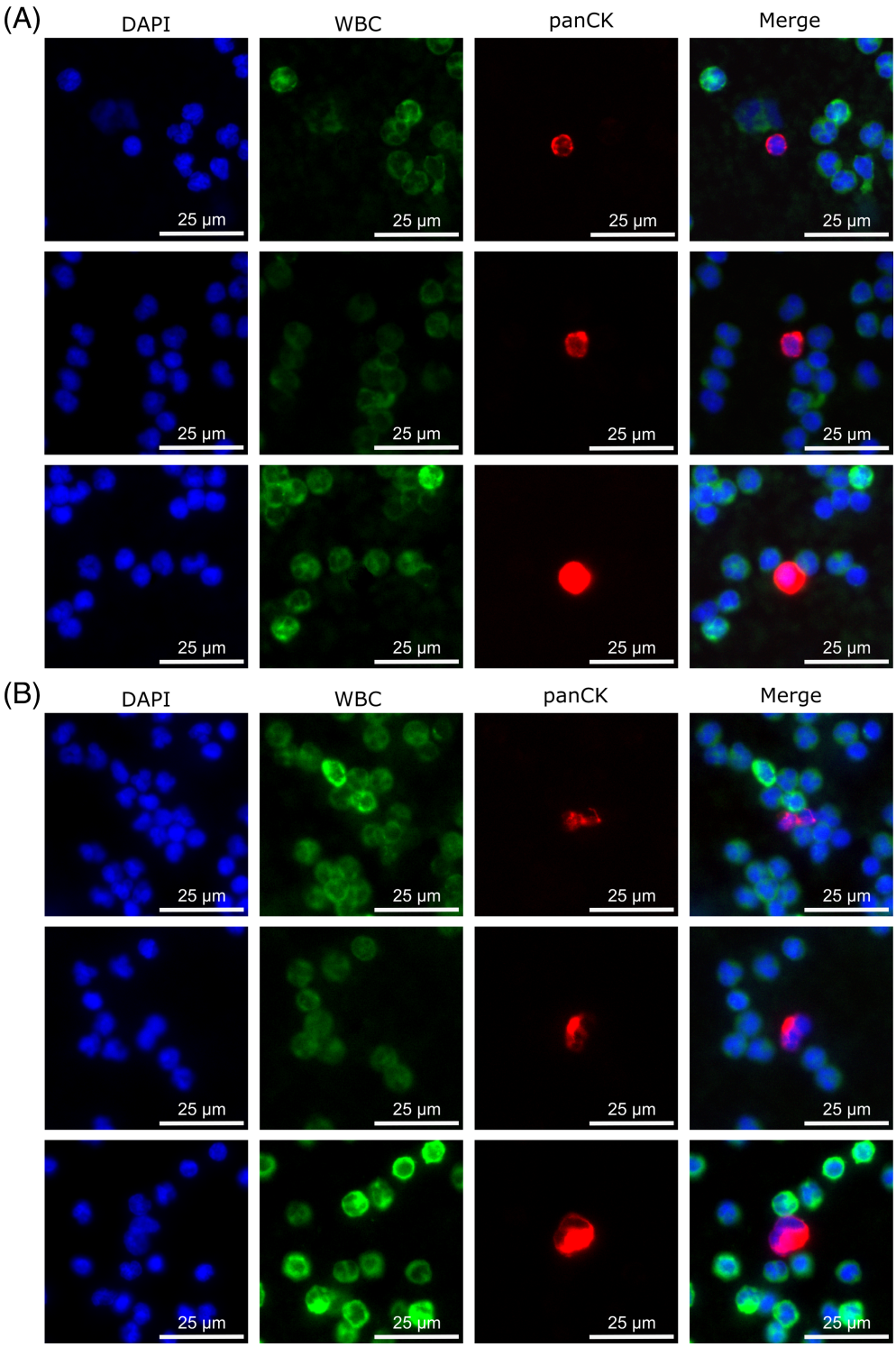

FIGURE 3 Example images of three different (A) CTC and (B) CTC duplets detected by immunofluorescent staining of panCK (red), white blood cell (WBC) (green) and nuclear counterstaining using DAPI (blue); A panCK positive, DAPI positive and WBC negative cell was defined as CTC.

study, all of the 12 samples positive without pre-amplification were positive for the neuroendocrine marker panel, whereas only five of them (41.7%) were positive for the epithelial marker panel. After the pre-amplification step, 39 of the 45 positive samples (86.7%) were positive for the neuroendocrine panel, 35 (77.8%) for the epithelial panel and 29 (64.4%) for both panels. After pre-amplification *DLL3* was detected in 9 (12.2%) samples, which were also positive for the epithelial and the neuroendocrine panel except for one single case (Figure 1).

3.2 | CTC-related gene transcripts after modified CTC enrichment

Next, we modified the enrichment of CTCs to investigate if a less stringent separation of target and background cells before the molecular analysis of the specified transcripts by qPCR, would finally increase the number of positive findings. For this purpose, we chose a microfluidic separation of the target cells using a PX GEN3D4.5

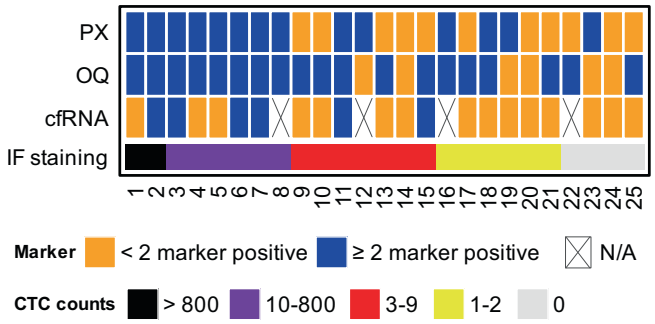

FIGURE 4 Heatmap showing SCLC patient sample (n = 25) overall CTC positivity after Parsortix (PX), OncoQuick (OQ) CTC enrichment or in circulating free RNA (cfRNA) detected by qPCR. For the definition of positivity, a threshold was set considering a minimum of two positive marker per patient sample. Samples with positive findings are marked blue and with negative findings orange. Samples not available are marked with an x. Below CTC counts of each sample detected by immunofluorescent (IF) staining are depicted. Samples with over 800 CTC are shown in black, 10-800 CTC in purple, 3-9 CTC in red, 1-2 CTC in yellow, 0 CTC in light grey.

cassette, and in parallel a OQ density gradient centrifugation, respectively, for an additional set of SCLC patient (n = 25) and HD (n = 11) blood samples. Among the samples enriched by microfluidics, 17 patient samples (68.0%) were assigned as positive due to the presence of at least one of the transcripts. The positivity rate of the neuroendocrine markers in these samples was 100%, while the epithelial marker panel in contrast contributed to just 76.5% of positive cases. After density gradient enrichment, 22 (88.0%) of the same 25 patient samples were positive by qPCR, with 86.4% of these being positive for neuroendocrine and 90.9% for the epithelial markers. Figure 2 depicts a heatmap of marker positivity in the individual patients with both isolation methods.

Overall, the two CTC isolation systems coincided to a large extent (PABAK $\kappa = 0.760$). However, at the individual marker level, a substantial level of concordance was only observed with the highly specific *DLL3* and *CK19* LC (Table S2) assays. The *CK19* LC hybridization probe RT-qPCR assay was introduced to minimize false positive samples, due to a very high specificity.[25] Compared to the *CK19* TaqMan assay, the *CK19* transcript was not amplified in any of the HD samples using the hybridization probe assay.

In order to minimize false positive patient samples due to threshold setting, we applied another more stringent threshold considering a minimum of two positive markers for overall positivity per sample. Thereafter still 11/25 (44.0%) of patient samples remained positive after PX and 16/25 (64.0%) after OQ enrichment.

3.3 | CTC-related gene transcripts in cfRNA

The presence of the CTC-related gene transcripts was additionally measured in cfRNA isolated from blood plasma. The plasma was obtained from 21 of the 25 SCLC blood samples, which had been processed using OQ density gradient centrifugation and the microfluidic GEN3D4.5 separation cassette. Thus, a direct comparison of the CTC-related transcripts in cfRNA and enriched cells was feasible. Overall, 11/21 (44.0%) patient samples were positive for at least one marker, and two HD samples were positive. The positivity of a minimum of two positive markers was less frequently observed in cfRNA (24.0% [6/21]) than in CTCs enriched by PX (44.0% [11/25]) or OQ (64.0% [16/25]).

3.4 | Immunofluorescent staining

In addition to the molecular analysis of CTC-related gene transcripts, the presence of CTCs was also assessed in blood samples of the same patient cohort (n = 25) enriched by a Ficoll-based density gradient centrifugation (CPT) using the Circulating Epithelial Cell nPAC™ RUO Kit (Axon Dx, LLC). At least one CTC, defined as nucleated CKpan positive and WBC negative cell, was detected in 84.0% of the 25 samples (Figure 3A). In seven patient samples CTC duplets were found (Figure 3B). More than 800 CTCs were observed in two patient samples (patient 1 and 2), more than three CTCs in 11 (44.0%), and more than two CTCs in 15 (60.0%) samples. All eight patient samples with the highest CTC counts (>10 CTCs) were also positive for at least two qPCR markers after CTC enrichment with PX and OQ. In addition, CTC duplets were detected in six (75%) of these eight patient samples. In all patient samples with CTC duplets, *DLL3* positivity was observed. Furthermore, 4/6 cfRNA positive cases were observed in the group of samples with high CTC load. All but one positive cfRNA samples were also positive after both PX and OQ CTC isolation (Figure 4).

3.5 | Association of CTC-related gene transcripts with patient characteristics

We analyzed the clinical data from 66 SCLC patients. From these we had 74 blood samples available, with 38 taken at primary diagnosis and 34 at progression (in two cases no information was available). A significant association was observed between the epithelial panel positivity and patient outcome, as well as of *DLL3* positivity and disease stage at blood draw (Fisher's exact test $p = 0.015$ and $p = 0.011$; Table S3).

From 8/66 patients, two blood samples were taken during the course of the disease, which allowed a longitudinal analysis (Figure S7). From five of them, one sample was obtained at primary diagnosis and one at progressive disease (patient 1-5). In addition, from two further patients two samples were taken at progression (patient 6-7). As from one patient, two blood samples were taken within a short time frame at primary diagnosis, this patient was not included in the longitudinal analysis. The specific marker *DLL3* was not detected at the first blood draw but in three patient samples taken at progressive disease (patient 2, 5 and 6). The epithelial and neuroendocrine marker panel were mostly either present at both investigated

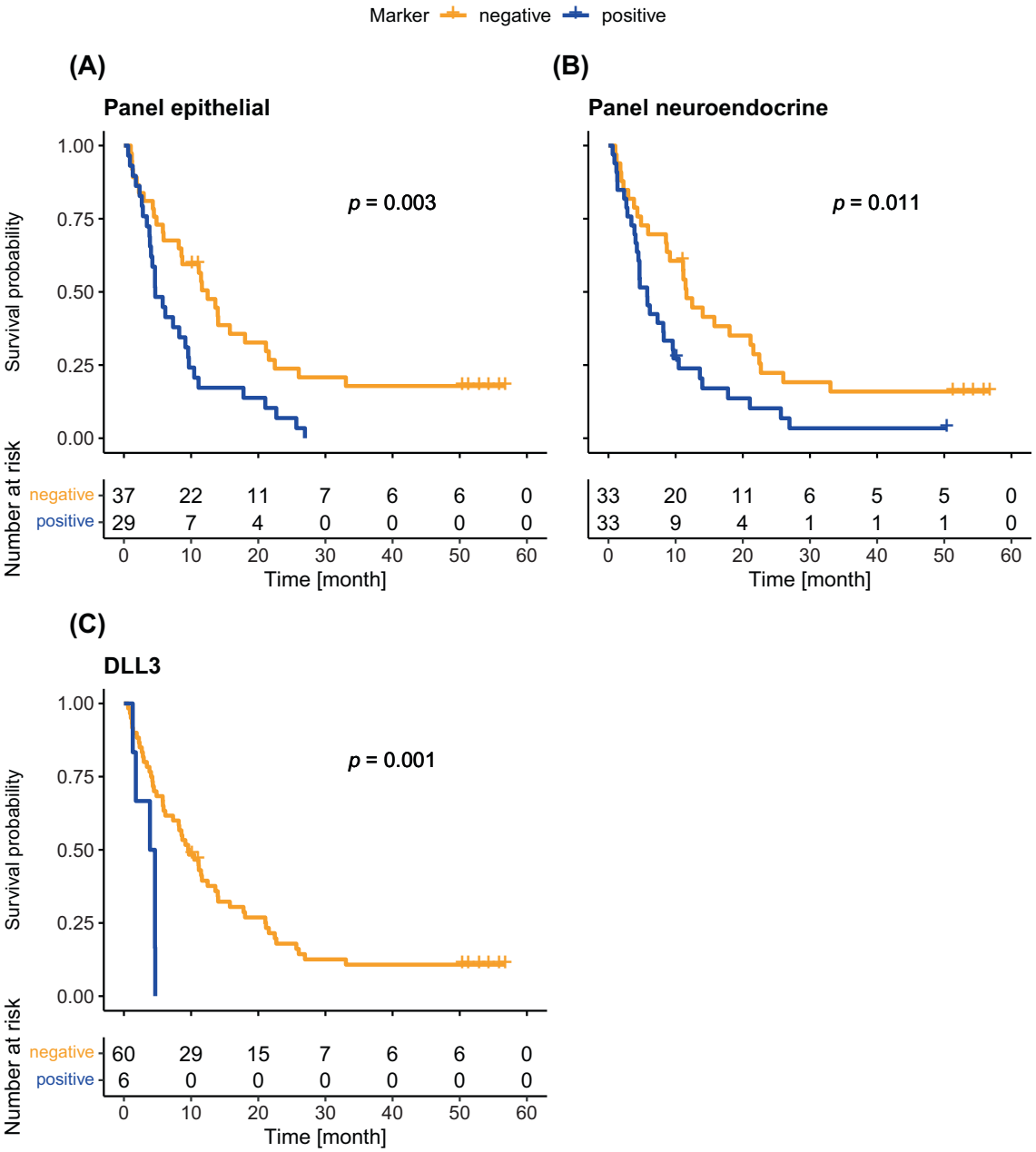

FIGURE 5 Kaplan-Meier plots for overall survival of small-cell lung cancer patients according to (A) epithelial (CK19, EpCAM) and (B) neuroendocrine (CHGA, SYP) marker panel and (C) DLL3 positivity (blue) and negativity (orange); Log-rank testing was used for comparing the patient's outcome; $p < .05$ is defined as level of significance; median follow-up: 52.9 months.

timepoints or only at further progression of the tumor (patient 2 and 7). In two patients, the epithelial (patient 1) or the neuroendocrine marker panel (patient 4) was only positive at the first blood draw. Due to a low expression of the marker with higher background expression (*EpCAM* and *SYP*), these samples might be borderline positives resulting from threshold setting. A single patient was negative for both panels and *DLL3* at both timepoints (patient 3), which might have generally low CTC numbers.

In addition, we analyzed the association of CTC-related transcripts and CTC counts detected in 25 blood samples (23 patients) which had been processed using the modified PX enrichment protocol and OQ (see Table S4). The presence of neuroendocrine transcripts was more likely in former than in current smokers (Fisher's exact test $p = 0.035$), while epithelial markers and *DLL3* were more often observed in patients who had already died at study completion (Fisher's exact test $p = 0.027$ and $p = 0.007$). Furthermore, *DLL3* presence was significantly more frequent in patients with extensive disease at primary diagnosis (Fisher's exact test $p = 0.028$). The presence of CTC at a threshold of at least two or five CTCs, or of CTC duplets was not associated with any of the patient characteristics. As

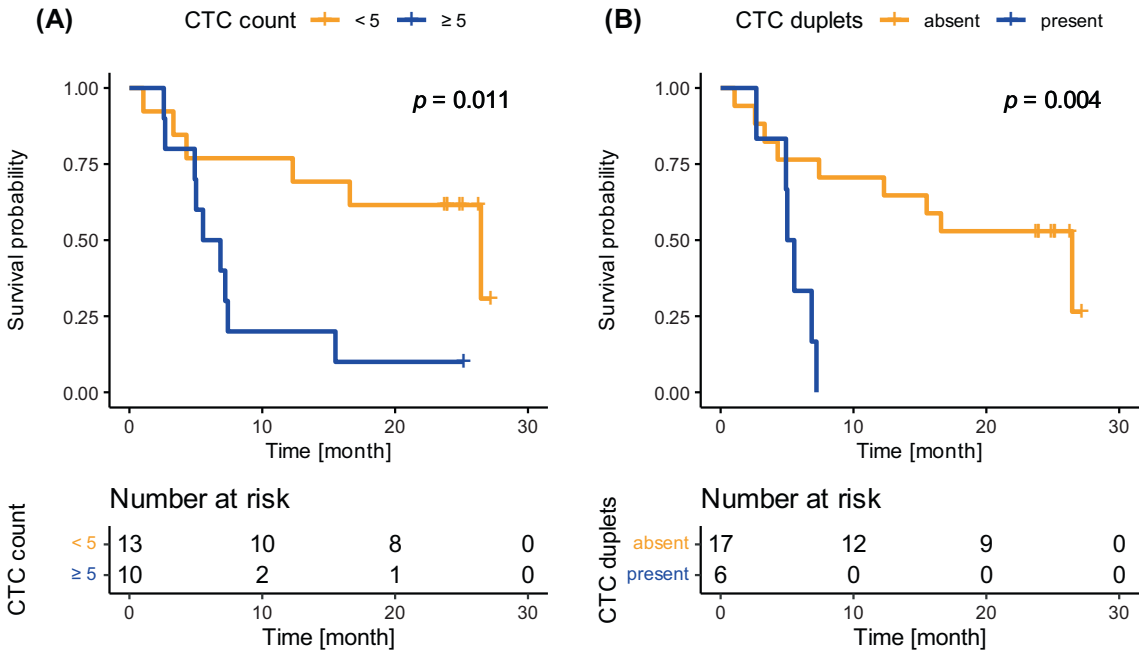

FIGURE 6 Kaplan-Meier plots for overall survival of small-cell lung cancer patients according to (A) ≥5 CTC (blue) or <5 CTC (orange) and (B) with present (blue) or absent (orange) CTC duplets; Log-rank testing was used for comparing the patient's outcome; $p < .05$ is defined as level of significance; median follow-up: 25.1 months.

the number of longitudinal blood samples was very small in this cohort (n = 2), we did not perform any additional analyses in these cases.

3.6 | Association of CTC-related gene transcripts and CTC counts with overall survival

In blood samples enriched with the PX GEN3D6.5 separation cassette, the positivity of each marker investigated by qPCR individually was associated with shorter OS of the 66 SCLC patients after pre-amplification (Figure S1). When combined into epithelial and neuroendocrine marker panel, both panels were also associated to shorter OS (neuroendocrine: median OS 5.2 vs 11.6 months; epithelial: median OS 4.7 vs 11.6 months) (Figure 5A, B). *DLL3* was also associated with shorter OS (Figure 5C). In the same samples without pre-amplification only the neuroendocrine panel was associated to shorter OS (median OS 4.4 vs 9.7 months, Figure S2).

In patient samples with PX enrichment using the GEN3D4.5 separation cassette, only the epithelial panel and *DLL3* was associated to shorter OS (both: median OS 6.9 months vs not reached, Figures S3 and S4). After OQ gradient centrifugation enrichment *DLL3* (median OS 7.0 vs 26.5 months) but none of the marker panels assessed in the CTCs was associated with shorter OS (Figures S5 and S6). cfRNA fraction analysis did not result in a significant association to OS (data not shown).

Similarly, the presence of at least five CTCs or CTC duplets detected by IF staining was associated with shorter OS of the 25 patients (CTCs: median OS 6.2 vs 26.5 months; CTC duplets: median OS 5.3 vs 26.5 months) (Figure 6A, B); no association was found when using a cut-off of three or one CTC (data not shown).

4 | DISCUSSION

Clinical characteristics such as disease stage, performance status, the presence of metastases, response to first line therapy, applied treatment lines and others provide significant information on patients' survival outcome.[29] Due to the fatal prognosis of SCLC patients, prognostic and predictive biomarkers are highly needed. Liquid biopsies may offer a favorable monitoring procedure to analyze biomarkers when no tissue biopsy is available or serial testing is required. The analysis of CTCs or cfRNA in the patients' blood may give clinically relevant information concerning prognosis, therapy decision or treatment response. Indeed, Hou et al[16] demonstrated the prognostic value of CTCs in SCLC independent from the clinical factors stated above. In our present study we aimed at defining optimal experimental conditions in terms of CTC recovery from peripheral blood of SCLC patients via the application of different CTC isolation and detection methods based on IF staining and qPCR of CTC-related transcripts including the predictive biomarker *DLL3*.

We were able to increase the positivity rate by 3.8-fold to 60.8% (45/74) due to the introduction of a pre-amplification prior to qPCR of CTC-related transcripts. Using the same transcripts in a further cohort of samples which had been enriched for CTC utilizing less stringent methodologies, the positivity rate was even higher, namely 88.0% (22/25) with density gradient centrifugation and 68.0% (17/25)

with a modified microfluidic enrichment. CTCs were detected in 84.0% of the cases (21/25) by IF staining in the peripheral blood from the same patient cohort.

Using the FDA approved CellSearch method previous studies achieved similar detection rates ranging from 73% to 90% in cohorts with 26 to 97 SCLC patients.[11–16,30] Shi et al[31] detected 94.5% SCLC patients based on density gradient centrifugation and CTC detection with *CK19* mRNA expression. Acheampong et al[18] also used the PX microfluidics platform with the 6.5 µm critical step size cassette to enrich CTC of 20 SCLC patients' blood and were able to detect them in 55.0% of cases by IF staining of the epithelial EpCAM and cytokeratins. In both studies that compared the positivity rate of the label-free PX and EpCAM-dependent CellSearch, a lower CTC yield was achieved by CellSearch; a possible explanation can be the downregulation of epithelial markers in some CTCs.[17,18] Besides the label-free enrichment of CTCs, PX offers the analysis of CTCs by qPCR due to the enormous reduction of white blood cells.[32] qPCR can assess a variety of markers in the same sample; the addition of neuroendocrine or therapy-relevant markers may therefore lead to a more comprehensive analysis of CTCs than by epithelial markers alone.

The neuroendocrine marker CD56 (NCAM1) was assessed in CTCs by Messaritakis et al[33]; however, the presence of *NCAM1* in healthy donor blood samples led us to the conclusion that this marker was less optimal for CTC analyses at the transcriptional level than the neuroendocrine *CHGA* and *SYP*.[19] Similar to that previous study,[19] *CHGA* and *SYP* were more often positive than the epithelial markers (*EpCAM* and *CK19*) in blood samples enriched by the standard microfluidic separation using the GEN3D6.5 cassette. All CTC-related transcripts were associated with shorter OS, both each transcript individually and combined as a neuroendocrine and epithelial panel. The contrary was observed in samples enriched by density gradient centrifugation. In these samples, the epithelial marker panel was more often detected than the neuroendocrine markers. A possible explanation can be the enrichment of different CTC phenotypes depending on the enrichment method.

Irrespective of the CTC enrichment procedure, *DLL3* was associated with shorter OS and thus is a strong predictor of patient survival. *DLL3* is part of the NOTCH signaling pathway and is an optimal CTC biomarker as it is not expressed in normal lung tissue. Furthermore, *DLL3* can be used as a target for antitumor drugs.[34] In previous clinical studies, the antibody-drug conjugate ROVA-T was evaluated.[35,36] Despite of the negative results of these studies, extensive further research has been undertaken to develop novel targeted therapies, for example CAR-T cells against *DLL3*,[37] photo-,[38] and radioimmunotherapy.[39] Recently presented results of the DeLLphi-330 study indeed demonstrated impressive antitumor activity of the bispecific T-cell engager AMG 757 (Tarlatamab) with durable responses and promising survival.[7]

Although, the detection of the CTC related transcripts in cfRNA resulted in a lower positivity rate than in CTC enriched samples, the concordance with PX and OQ positivity was high. The lower positivity rate might be explained by generally lower cfRNA yield, also indicated by a low recovery of a spiked-in template (data not shown).[24] The evaluation of other cfRNA isolation procedures in a larger cohort might be useful to increase RNA yields and underline the prognostic relevance of cfRNA in SCLC, which has previously been shown in NSCLC.[40]

In addition to the molecular analysis of the CTC-related markers, we evaluated the novel Circulating Epithelial Cell nPAC RUO Kit including label-free enrichment and IF staining for the enumeration of epithelial-like CTCs. CTCs were found in the majority of the samples, and a concordance of high CTC counts, and RNA marker positivity was observed (Figure 4). In line with other studies,[16,30] the incidence of five or more CTCs and the presence of CTC duplets was significantly associated with shorter OS.

Therefore, in our study both with IF staining and with qPCR analysis of liquid biopsies, patients at risk for shorter overall survival could be identified. Both methods have their assets and drawbacks. CTC enumeration reveals prognostic information, as indicated by our study. The analysis with qPCR allows testing for multiple transcripts at once, including predictive biomarker and relevant drug targets such as *DLL3*. An important prerequisite for CTC analysis by qPCR is the efficient depletion of leukocytes as achieved by the microfluidic GEN3D6.5 separation cassette. Enrichment strategies using PX cassettes having a critical step size of just 4.5 µm or OQ density gradient centrifugation result in a higher number of contaminating leukocytes and probably of CTCs of smaller cell sizes. However, not all of the patients positive for individual markers after OQ or PX 4.5 µm CTC enrichment have worse overall survival. Setting an appropriate cut-off threshold to identify patients with worse outcome is a critical point not only in qPCR-based detection of CTCs, but also in the detection by immunofluorescent staining.

A limitation of our study is the lack of a comparison between the microfluidic enrichment using the standard GEN3D6.5 separation cassette and IF staining due to small patient blood volume. High CTC counts (Figure 6) and marker positivity after modified enrichment (Figures S3, S4 and S6) were associated with patient survival; however future studies with larger patient cohorts and serial blood draws at clinically relevant events are needed to verify the results. Although in a small subset of patients, we were able to analyze the gene expression of the epithelial and neuroendocrine panel and of *DLL3* in two serial blood samples. As already indicated by the survival analysis, *DLL3* was only present at disease progression, but not at primary diagnosis (see Figure S7). While we evaluated the CTC-related transcripts during the course of the disease and found that, for example, *DLL3* could indicate progression of the disease, Zhang et al[41] could link the dynamic changes in CTC numbers and SLNF11 protein expression to treatment response verified by imaging. Longitudinal monitoring of CTC-related transcript could potentially be used to monitor SCLC patients and guide treatment decisions based on the observed disease status.

To conclude, our results indicate the necessity of a pre-amplification step for the detection of CTC-related transcripts using qPCR in order to maximize the number of positive findings in SCLC. The choice of CTC enrichment is largely dependent on the specific clinical question. On the one hand, a high CTC load detected by immunofluorescent staining might indicate disease progression and

identify patients with poor outcome who could benefit from further clinical intervention. On the other hand, the molecular characterization of neuroendocrine-like or epithelial-like CTCs as well as of druggable targets is possible using qPCR. This may aid in the differential diagnosis of SCLC and in guiding treatment decisions. For this purpose, the use of the GEN3D6.5 microfluidic CTC enrichment is favorable to eliminate leukocyte background. In addition to CTC analysis, cfRNA may be a promising simplified tool to analyze a variety of relevant transcripts in cancer patients.

Due to the high CTC load in the blood of SCLC patients, liquid biopsy analysis using CTC offers a tremendous potential, from which patients could benefit in terms of diagnosis or prediction. In the future, molecular profiling of CTC on the RNA and protein level performed regularly as timely monitoring of patients, will reveal the presence of targetable biomarkers, as well as facilitate the early detection of subpopulations of tumor cells that might be linked to resistance to treatment or disease progression.

AUTHOR CONTRIBUTIONS

Eva Welsch: conceptualization, methodology, formal analysis, visualization; writing—original draft; Barbara Holzer: investigation, writing—review & editing; Eva Schuster: investigation, writing—review & editing; Hannah Fabikan: resources; Christoph Weinlinger: resources; Elisabeth Hauptmann-Repitz: resources; Oliver Illini: resources, writing—review & editing; Maximilian J. Hochmair: resources, writing—review & editing; Michael B. Fischer: resources, writing—review & editing; Esther Weiss: investigation; Robert Zeillinger: supervision, writing—review & editing, project administration; and Eva Obermayr: supervision, project administration, writing—original draft. All authors agreed to the final version of the manuscript. The study reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST STATEMENT

Hannah Fabikan received speaker fee from Roche. Oliver Illini received speaker fees and/or honoraria for advisory boards from Boehringer Ingelheim, Eli Lilly, Menarini, Merck Sharp & Dohme, Pfizer, and Roche. Received research grants (institute) from Amgen outside of the submitted study. Maximilian Hochmair received honoraria from AstraZeneca, Amgen, Bristol-Myers Squibb, Boehringer Ingelheim, Merck Sharp & Dohme, Pfizer, and Roche, and has had consulting or advisory roles with Boehringer Ingelheim, Merck Sharp & Dohme, Pfizer, Novartis, and Roche. Eva Welsch, Barbara Holzer, Eva Schuster, Christoph Weinlinger, Elisabeth Hauptmann-Repitz, Michael B. Fischer, Esther Weiss, Robert Zeillinger, and Eva Obermayr declare no conflicts of interest.

ETHICS STATEMENT

The study was approved by the Ethic Committee of the Medical University of Vienna, Austria (EK366/2003 and EK2266/2018) and followed guidelines of good scientific practice. All patients and HD signed an informed consent transfer agreement to participate in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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