

# Development of an Electrochemical CCL17/TARC Biosensor toward Rapid Triage and Monitoring of Classic Hodgkin Lymphoma

Christopher Rinaldi,\* Damion K. Corrigan,<sup>†</sup> Lynn Dennany,<sup>†</sup> Ruth F. Jarrett, Annette Lake, and Matthew J. Baker<sup>†</sup>



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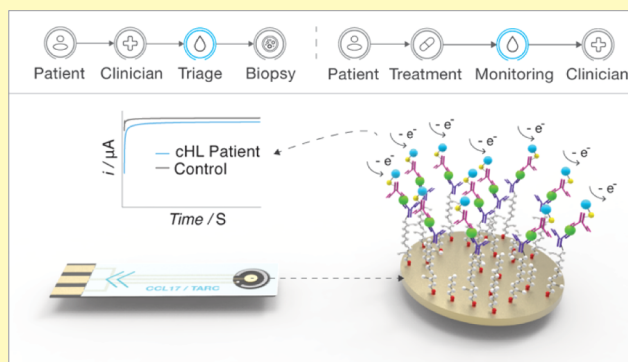
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Supporting Information

**ABSTRACT:** A point-of-care blood test for the detection of an emerging biomarker, CCL17/TARC, could prove transformative for the clinical management of classic Hodgkin lymphoma (cHL). Primary care diagnosis is challenging due to nonspecific clinical presentation and lack of a diagnostic test, leading to significant diagnostic delays. Treatment monitoring encounters false-positive and negative results, leading to avoidable chemotherapy toxicity, or undertreatment, impacting patient morbidity and mortality. Here, we present an amperometric CCL17/TARC immunosensor, based on the utilization of a thiolated heterobifunctional cross-linker and sandwich antibody assay, to facilitate novel primary care triage and chemotherapy monitoring strategies for cHL. The immunosensor shows excellent analytical performance for clinical testing; linearity ( $R^2 = 0.986$ ), detection limit (194 pg/mL), and lower and upper limits of quantitation (387–50 000 pg/mL). The biosensor differentiated all 42 newly diagnosed cHL patients from healthy volunteers, based on serum CCL17/TARC concentration, using blood samples collected prior to treatment intervention. The immunosensor also discriminated between paired blood samples of all seven cHL patients, respectively, collected prior to treatment and during chemotherapy, attributed to the decrease in serum CCL17/TARC concentration following chemotherapy response. Overall, we have shown, for the first time, the potential of an electrochemical CCL17/TARC biosensor for primary care triage and chemotherapy monitoring for cHL, which would have positive clinical and psychosocial implications for patients, while streamlining current healthcare pathways.

**KEYWORDS:** electrochemistry, electrochemical immunosensor, biosensor, Hodgkin lymphoma, CCL17/TARC



An electrochemical CCL17/TARC immunosensor for primary care triage and secondary care chemotherapy monitoring would represent a paradigm shift in the clinical management of classic Hodgkin lymphoma (cHL). Recently, the chemokine, CCL17/TARC, has emerged as a highly promising candidate blood biomarker for cHL, which could be utilized for early diagnosis, monitoring treatment response, and early detection of relapse. Presently, no point-of-care blood tests exist for cHL in primary or secondary care. A primary care CCL17/TARC test would promote early detection of difficult to diagnose adolescent and young adult patients, enabling timely secondary care referral and accelerated diagnosis with core or excision biopsy. A CCL17/TARC test in oncology and hematology clinics would permit blood-based treatment response monitoring, enabling next-day clinical decisions prior to chemotherapy cycles.

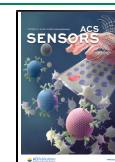
cHL is a B-cell-derived malignancy characterized by Hodgkin and Reed-Sternberg cells, the tumor cells, in a background of inflammatory cells. The interaction between Hodgkin and Reed-Sternberg cells and inflammatory cells is crucial to cHL pathogenesis. CCL17/TARC is highly ex-

pressed by Hodgkin and Reed-Sternberg cells and most likely plays a role in attracting T helper 2 (Th2) cells into the tumor microenvironment.<sup>1,2</sup> CCL17/TARC is elevated in  $\geq 90\%$  of untreated cHL patient serum samples relative to healthy persons,<sup>3,4</sup> and correlates with tumor burden,<sup>4–6</sup> and Ann Arbor stage.<sup>3,5,7,8</sup> Moreover, CCL17/TARC falls rapidly in response to successful treatment.<sup>4</sup>

In the United Kingdom (U.K.),  $\sim 2100$  individuals are diagnosed with Hodgkin lymphoma (HL) per year,<sup>9</sup> which represents  $\sim 10\%$  of all lymphoma cancers,<sup>10–12</sup> and  $>90\%$  of these are cHL.<sup>11,13</sup> cHL has a bimodal age-specific incidence curve with one peak for young adult (15–34 years) and elderly ( $\geq 50$  years) patient groups, respectively. In industrialized countries, cHL is the commonest malignancy in persons aged

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**Figure 1.** Product concept for the proposed electrochemical sensor and overview of the developed sandwich immunoassay.

15–24 years. Patients often present with painless cervical lymphadenopathy, symptoms related to enlarged mediastinal nodes, such as dry cough or shortness of breath,<sup>11,12,14</sup> or systemic symptoms such as fever, night sweats, and weight loss (B symptoms). Itchy skin and alcohol-induced lymph node pain may also be present.<sup>14–16</sup>

Nonspecific clinical presentation and lack of a diagnostic test cause diagnostic delays. Timely diagnosis is a particular challenge in patients <40 years of age; only 0.4% of patients who present to primary care with lymphadenopathy will have cancer.<sup>17</sup> National Institute for Health and Care Excellence (NICE) guidelines recommend referral of symptomatic patients through the “two-week wait” cancer pathway;<sup>18</sup> however, only one-third access diagnosis through this pathway, while almost one-fifth access emergency settings.<sup>19,20</sup> Patients diagnosed in emergency settings are more likely to have an advanced-stage disease, with poorer 3 year relative survival compared to patients diagnosed through nonemergency routes.<sup>19</sup> Delayed diagnosis has negative psychosocial effects for patients and families; these data further suggest that delayed diagnosis leads to inferior clinical outcomes. Hence, there is a strong argument for primary care triage to accelerate patient referral.

CHL has a good prognosis with cure rates of >90% in patients with the limited-stage disease and ~70% in patients with advanced-stage disease.<sup>21</sup> Treatment aims to cure CHL while minimizing side effects, which cause significant patient morbidity and mortality, since most are young at diagnosis.<sup>21</sup> Treatment protocols now incorporate response-adapted therapy where interim fluorodeoxyglucose (18F) positron emission tomography, coupled with computerized tomography (PET-CT), is used to assess treatment response after two or three chemotherapy cycles to guide treatment decisions. Although PET-CT has improved outcomes, it is not perfect.<sup>22</sup> False-positive results lead to avoidable, delayed toxicity,<sup>8,23,24</sup> and false-negative results lead to undertreatment and disappointing outcomes.<sup>8</sup> Hence, more sensitive markers are needed to evaluate therapeutic response.<sup>22</sup> Pretreatment

CCL17/TARC falls rapidly with successful treatment response, and interim CCL17/TARC may better assess monitoring compared to PET/CT.<sup>5,25</sup> CCL17/TARC may inform treatment response after one cycle of chemotherapy,<sup>5,25</sup> allowing earlier identification of refractory patients compared to PET-CT. Hence, a CCL17/TARC test may be useful as an adjunct or alternative to PET-CT to improve risk-adapted therapy.

Electrochemical immunosensors permit characterization of antibody–antigen interactions into quantifiable electrical signals. The technology has the potential to provide rapid, economical point-of-care clinical testing.<sup>26,27</sup> Thus far, diverse approaches have been reported for electrochemical biomarker detection, outlined in review articles.<sup>28–32</sup> Previously, we developed an “unlabeled” electrochemical immunosensor for TREM-1, MMP-9, HSL, and IL-6 biomarkers.<sup>33</sup> However, sandwich-based “labeled” strategies improve assay sensitivity and reproducibility for clinical testing, by overcoming nonspecific adsorption of biomolecules.<sup>26</sup> Sandwich-based electrochemical biosensors have demonstrated the detection of cancer biomarkers, such as prostate-specific antigen,<sup>34</sup>  $\alpha$ -fetoprotein,<sup>35</sup> CA19-9,<sup>36</sup> HER2,<sup>37</sup> and CEA.<sup>38</sup> Recently, micro- and nanotechnology and sophisticated surface chemistries have increasingly been utilized to improve test performance. The Estrela group reported an electrochemical biosensor that utilized carboxylic functionalized polypyrrole films of nanometer thickness on microarray electrodes for blood testing.<sup>39</sup> The Ingber group developed an antifouling coating, which featured a network of nanomaterials, to reduce nonspecific adsorption of biomolecules for anti-interleukin 6 detection in blood.<sup>40</sup> The Morgan group also demonstrated a fully integrated microfluidic electrochemical system for blood cytokine detection.<sup>41</sup> Critically, however, clinical acceptance and adoption of electrochemical biosensors for clinical testing remain elusive, particularly for cancer diagnostics.

Principally, there is an urgent need to translate proof-of-principle studies on spiked samples,<sup>34,35,37,38</sup> into clinical feasibility studies with cancer patients to validate performance. Proposed electrochemical strategies must also be conducive to

commercialization to promote clinical adoption and minimize costs per test for healthcare. Thus, we propose robust clinical testing of an electrochemical CCL17/TARC sandwich immunosensor on cHL patients. Our strategy shall focus on the implementation of standard electrode architectures and simple functionalization chemistries, which hold potential for commercialization and rapid and economical testing with printed electrodes (Figure 1). Only in doing so will clinical translation become a reality, which would transform the patient pathway and positively impact outcomes for cHL patients.

## MATERIALS AND METHODS

**Clinical Samples.** Pre- and on-treatment cHL patient samples were collected from two studies: Investigation of The Cause of Hodgkin lymphoma (ITCH) and Biomarkers and classical Hodgkin lymphoma (BACH). Samples represent serum CCL17/TARC levels across cHL patients, which were quantified using the Human CCL17/TARC Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis). Healthy volunteer samples were collected from the Study of Healthy Adult Response to EBV (SHARE). Fifty-four samples were tested: 5 healthy volunteers, 42 pre-, and 7 on-treatment cHL patients (details in Table S1). All studies were approved by Research Ethics Committees, and all participants gave written, informed consent.

**Materials and Reagents.** Concentrated sulfuric acid (98%), hydrogen peroxide (30%) in water, and the CHI120 polishing kit (CH Instruments, Inc., Austin) were purchased for electrode cleaning. Sulfosuccinimidyl 6-(3'-(2-pyridylthio)propionamido)hexanoate (Sulfo-LC-SPDP), DL-dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), tris(2-carboxyethyl)phosphine (TCEP), phosphate-buffered saline (PBS), deionized (DI) water (Millipore, Livingston, West Lothian), Human CCL17/TARC DuoSet ELISA (R&D Systems, Inc., Minneapolis), and Amicon Ultra-0.5 mL 3 kDa filters were used for immobilization. Potassium ferri/ferrocyanide and the DuoSet ELISA Reagent kit 1 (R&D Systems) were purchased for testing. Unless otherwise stated, reagents were purchased from Sigma-Aldrich (Sigma-Aldrich, Poole, Dorset).

**Instrumentation.** Experiments were conducted on a PalmSens Emstat potentiostat (PalmSens, The Netherlands) with one CH Instruments CHI101 gold working, CHI111 silver silver/chloride reference, and CHI115 platinum counter electrode.

**Cleaning.** Working electrodes were chemically, mechanically, and electrochemically cleaned as documented in the Supporting Information.

**Immobilization.** A sandwich CCL17/TARC antibody assay was immobilized on working electrodes through modification of a previous protocol.<sup>33,42</sup> All details are provided in the Supporting Information.

Amperometric experiments applied a potential of  $-0.2$  V for 120 s with 0.1 s time intervals. The time period produced the lowest standard deviation for triplicate measurements compared to shorter time periods. We hypothesize an extended time period ensures sufficient time for the signal to fully permeate the dense SAM antibody film. However, there may be scope to reduce this time period in the future.

**Assay Characterization.** Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were performed to characterize immobilization. Electrodes were immersed in 10 mM ferri/ferro potassium cyanide in 1× PBS solution. EIS measurements were recorded at open circuit potential (OCP) with an applied AC potential of 10 mV rms amplitude over a 0.1–100 000 Hz range. The measurement solution contained equal concentrations of potassium ferri/ferrocyanide and, thus, a potential was fixed between the two peaks of the redox couple. This made it possible to perform EIS measurements at OCP with theoretically equal amounts of oxidized and reduced species present before imposing the AC excitation signal. This is an approach that has been used by ourselves,<sup>42,43</sup> and many others previously. CV experiments were conducted between  $-0.4$  and  $0.6$  V with a step of 0.01 V at scan rates of 50 mV/s. All

measurements were performed on three functionalized working electrodes.

**Reproducibility Study.** Please refer to the Supporting Information for reproducibility study details on functionalized working electrodes.

**Statistics.** EIS data was modeled with Randles equivalent circuit and fitted with the Levenberg–Marquardt model to determine  $R_{ct}$  at working electrodes.  $R_{ct}$  variability was quantified for immobilization steps with probability distribution plots at 95% confidence intervals.

One-way analysis of variance (ANOVA) evaluated mass-transfer limited currents at 120 s for all samples tested during spiked studies at 95% confidence intervals. The test permits equality testing between two or more means and, thus, determines statistical significance between spiked CCL17/TARC and control samples. Post-hoc Tukey testing identified all statistically significant CCL17/TARC concentrations, where  $p$ -values  $< 0.05$ . Four-parameter logistic (4PL) regression determined the relationship between logarithms of CCL17/TARC concentration and current for statistically significant concentrations, where  $p$ -values  $< 0.05$ . 4PL regression is used to characterize nonlinear biological assays over wide concentration ranges and was recommended by the supplier. The model used the equation

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{(\log x_0 - x)p}}$$

where  $y$  is the response variable,  $A_1$  is the smallest response,  $A_2$  is the greatest response,  $x_0$  is the inflection point, and  $p$  is the slope parameter that defines curve steepness. Curve fitting used least squares regression and assumed a normal data distribution. No weighting was used as standard deviation was not proportional to CCL17/TARC concentration.

One-way ANOVA studies were conducted on currents at 120 s for pretreatment samples to determine statistical significance between respective cHL and control patients at 95% confidence intervals. One-way ANOVA also analyzed responses of patients categorized according to known CCL17/TARC concentration: control ( $< 1$  ng/mL), group A cHL ( $1-5$  ng/mL), group B cHL ( $5-10$  ng/mL), group C cHL ( $10-50$  ng/mL), and group D cHL ( $> 50$  ng/mL). This allowed statistical testing to determine whether electrochemical responses of patients were proportional to CCL17/TARC concentration. Tukey testing identified all statistically significant groups, where  $p$ -values  $< 0.05$ .

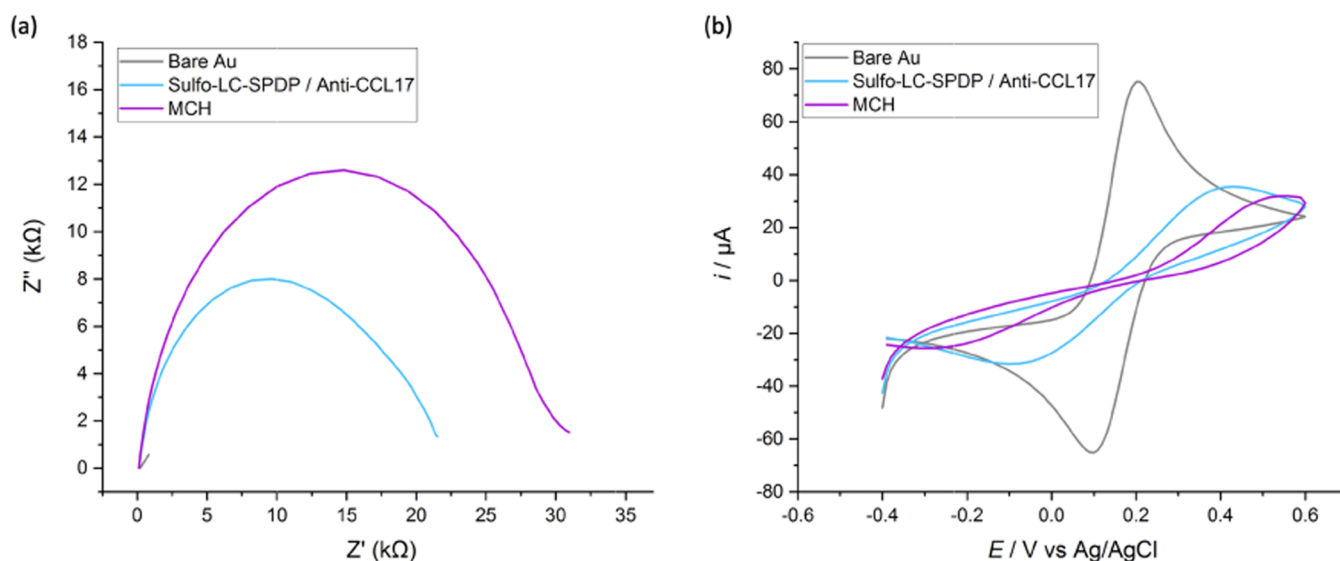
A paired  $t$ -test evaluated current responses at 120 s between all paired pre- and on-treatment patient samples at 95% confidence intervals. The paired  $t$ -test assumed the dependent variable was continuous, approximately normally distributed, did not feature outliers, and response measurements were independent. One-way ANOVA then determined across all individual patients whether pre- and on-treatment currents at 120 s were statistically significant at 95% confidence intervals. Tukey testing identified all statistically significant differences between groups where  $p < 0.05$ .

One-way ANOVA analyses assumed that the dependent variable was continuous, approximately normally distributed, the variance was approximately equal across groups, and responses were independent observations. Tukey testing used identical assumptions and assumed that responses were independent within and among groups. Tukey testing performs all pairwise comparisons and was appropriate given a large number of groups in our data while minimizing type I error.

The correlation between electrochemical and colorimetric ELISA tests was established by plotting the logarithm of predicted serum CCL17/TARC concentration for all patients. Linear regression determined the relationship between tests, and Pearson's  $r$  coefficient specified the magnitude of the relationship. Linear regression assumed linearity, the dependent variable was normally distributed, variance of error was constant, and observations were independent.

## RESULTS AND DISCUSSION

**Electrochemical Characterization.** Working electrodes were electrochemically cleaned (Figure S1) prior to immobi-



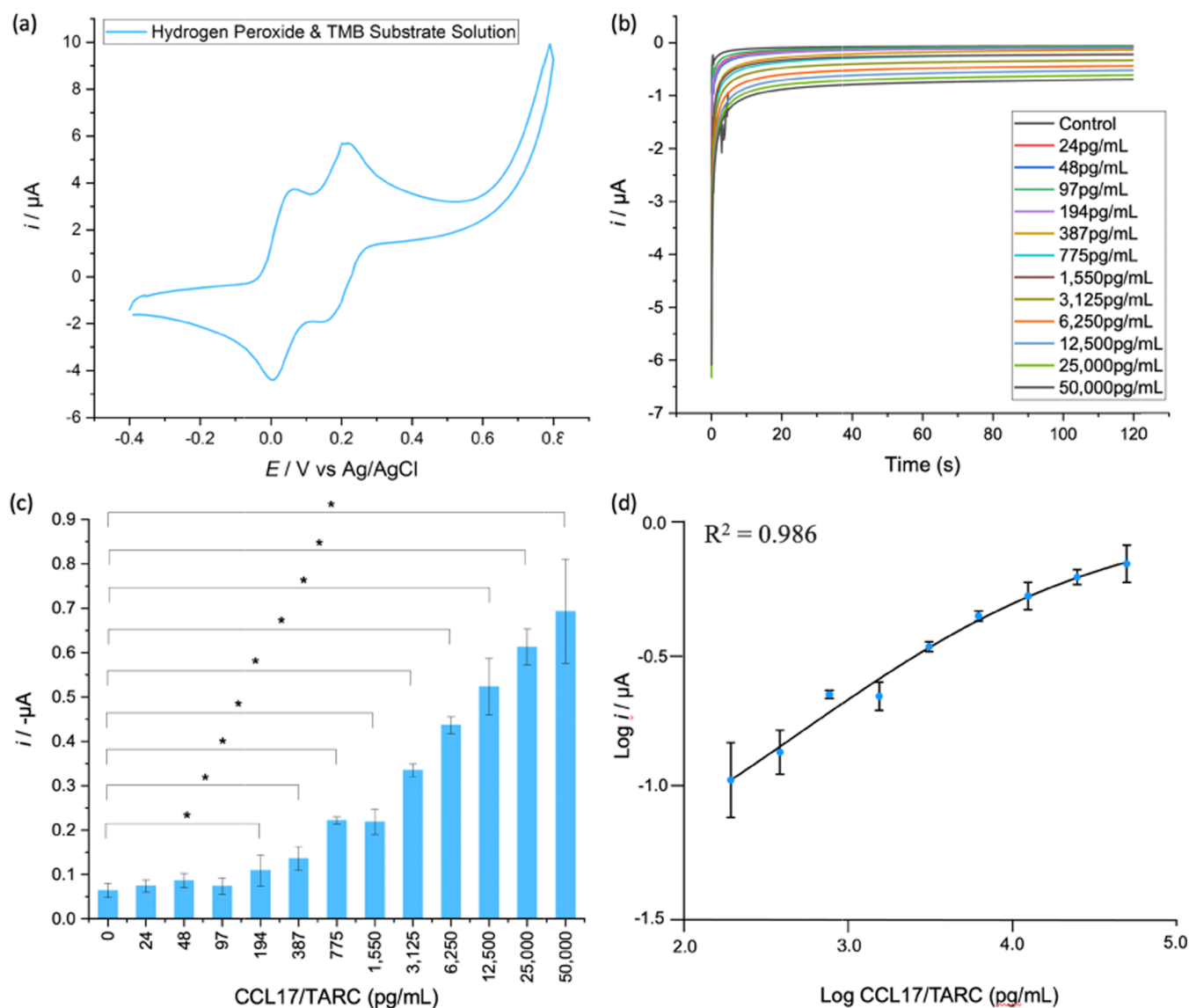
**Figure 2.** (a) Nyquist and (b) CV plot for one functionalized electrode following respective assay depositions.

lization to improve chemisorption between the cross-linker and gold. EIS and CV experiments determined electrode cleanliness (Figure 2). Bare working electrodes measured small charge-transfer resistances,  $R_{ct} = 107 \pm 11.5 \Omega$ , which indicates that the redox couple can freely participate in electron-transfer events, and implies that electrodes are appropriately clean. Working electrodes immobilized with primary CCL17/TARC antibodies conjugated to Sulfo-LC-SPDP measured a marked increase in  $R_{ct} = 2.173 \times 10^4 \pm 2.32 \times 10^3 \Omega$ , which suggests  $[\text{Fe}(\text{CN})_6]^{-3/4}$  ions have reduced ability to participate in electron transfer due to antibody SAM formation. Working electrodes functionalized with MCH measured a marked increase in  $R_{ct} = 2.95 \times 10^4 \pm 3.02 \times 10^3 \Omega$ , indicative of chemisorption of MCH to gold. Variation in  $R_{ct}$  was consistent across respective assay depositions, with a CV of 10.7% for the cross-linked CCL17/TARC antibody layer and 10.3% for the MCH layer, compared to 10.8% for bare electrodes. Hence, assay immobilization did not negatively influence measurement variation. Probability distribution plots quantified  $R_{ct}$  variability for all immobilization steps (Figure S2). Reassuringly,  $R_{ct}$  values were within 95% confidence intervals for all immobilization steps, indicative of reproducible SAM formation. Probability distributions and CV values may be employed as future quality control measures to ensure SAM formation gives rise to acceptable variation in  $R_{ct}$  at working electrodes. Additionally, a one-sample  $t$ -test should be employed to quantify SAM variation by comparing the population mean of  $R_{ct}$  for respective assay depositions to the hypothesized mean stated above.

Bare working electrodes measured  $I_{pa}/I_{pc}$  values close to unity, characteristic of a reversible redox reaction, and was anticipated for  $[\text{Fe}(\text{CN})_6]^{-3/4}$  species as it is a well-established reversible redox couple. Electrodes functionalized with CCL17/TARC antibodies measured decreases in  $I_{pa}$  and  $I_{pc}$ , which indicates that the dense protein layer hinders electron transfer of  $[\text{Fe}(\text{CN})_6]^{-3/4}$  ions at electrode surfaces. Electrodes immobilized with MCH observed decreases in  $I_{pa}$  and  $I_{pc}$ , which suggests thiol attachment further restricts electron-transfer events. Working electrodes recorded consecutive increases in  $\Delta E_p$  following assay depositions, from  $\Delta E_p =$

0.110 V to  $\Delta E_p = 0.809$  V.  $\Delta E_p$ , referred to as peak-to-peak separation, is defined as the separation between anodic and cathodic peak potentials.  $\Delta E_p$  is used to determine electrochemical reversibility and describes the rate of electron transfer between the working electrode and analyte. Electrochemically reversible reactions have a low thermodynamic barrier to electron transfer, resulting in a fast rate of electron transfer. Theoretically, electrochemical reversibility occurs when  $\Delta E_p = 57$  mV for a one-electron transfer reaction at 25 °C; however,  $\Delta E_p$  is typically higher during experimentation. Electrochemically irreversible reactions have a high thermodynamic barrier to electron transfer with a slow rate of electron transfer. Consequently, one must supply greater positive and negative potentials to initiate oxidation and reduction reactions, respectively, resulting in a greater  $\Delta E_p$ . Hence, increases in  $\Delta E_p$  for assay depositions indicate slow electron-transfer kinetics due to successful immobilization to working electrodes.

Assay reproducibility was evaluated over a 7 day period to determine SAM variability between electrodes and assay batches (Figure S3). Primary antibody immobilization increased  $R_{ct}$  signals at working electrodes on respective days. However,  $R_{ct}$  varied between working electrodes each day, with an interassay coefficient of variation (CV) of 21.7% over a 7 day period. Working electrodes also recorded variation in  $R_{ct}$  on different days, indicative of inconsistent antibody immobilization between assay batches. Working electrodes observed increases in  $R_{ct}$  following backfilling procedures on respective days. However,  $R_{ct}$  signals varied between working electrodes each day, with an interassay CV of 20.0% over the 7 day period, highlighting variation in MCH immobilization. Working electrodes also recorded variation in  $R_{ct}$  on different days, suggestive of inconsistent MCH immobilization between assay batches. Thiol SAM formation on gold occurs in a two-step process, a fast and slow growth phase, where the density and packing of molecules is influenced by several external and internal factors. The fast growth phase is influenced by surface cleanliness, concentration of adsorption molecules, immersion time, temperature, and humidity.<sup>44,45</sup> Fluctuations in experimental conditions may likely explain  $R_{ct}$  variability between days. The slow growth phase partly determines SAM



**Figure 3.** (a) CV plot of electrochemical characterization of TMB. (b) Amperometry plot of spiked CCL17/TARC samples (0–50 000 pg/mL) ( $n = 3$ ). (c) Plot of currents at 120 s for spiked CCL17/TARC samples ( $n = 3$ ). Asterisks indicate statistical significance, where  $p < 0.05$ . (d) Calibration curve of the logarithm of CCL17/TARC concentration and logarithm of statistically significant currents at 120 s, where  $p < 0.05$  ( $n = 3$ ). The 4PL regression model used the equation:  $y = A_1 + \frac{A_2 - A_1}{1 + 10^{(\log x_0 - x)p}}$ , and best fit values were calculated as  $A_1 = -1.482$ ,  $A_2 = 1.889 \times 10^{-2}$ ,  $\log x_0 = 2.863$ , and  $p = 0.4816$ .

morphology and is influenced by hydrogen bonding and van der Waals forces between alkane thiol chains.<sup>46</sup> Thus, SAM stability and density may be improved by selecting alkane thiols of increased chain length. Nevertheless, current findings indicate successful assay immobilization.

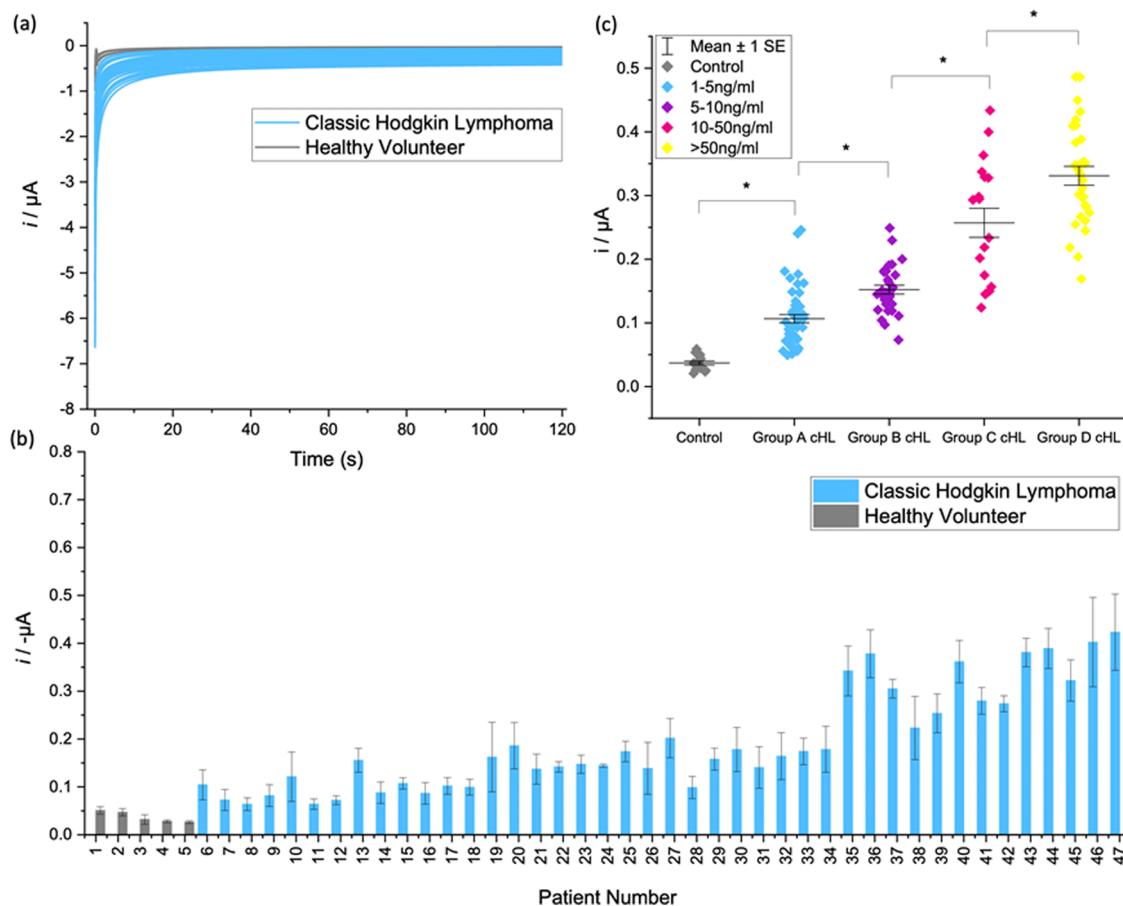
#### Electrochemical Detection of TMB Oxidation States.

Electrodes were immersed in substrate solution and subjected to CV to determine the redox potentials of TMB products. In sandwich assays that utilize horseradish peroxidase, the enzyme catalyzes the reduction of hydrogen peroxide to water in the presence of the native TMB diamine that acts as a proton donor.<sup>47</sup> Consequently, TMB is subjected to a one-electron transfer oxidation reaction. The oxidized TMB product can be detected electrochemically at  $E_{pa} = 0.06$  V (Figure 3a). The TMB product often undergoes a second oxidation reaction in the presence of sulfuric acid and can be detected electrochemically at  $E_{pa} = 0.21$  V. The TMB products gain one

electron during subsequent reduction reactions, which were observed at  $E_{pc} = 0.17$  V and  $E_{pc} = 0.01$  V, respectively. Hence, electrodes identified redox reaction characteristic of electrochemical TMB detection.<sup>48</sup> The oxidized product is proportional to antigen concentration and can be measured electrochemically by reducing oxidized species with an appropriate  $E_{pc}$ . A potential of  $E_{pc} = -0.20$  V was selected for amperometric experiments as the potential does not coincide with oxidation potentials,  $E_{pa}$  of TMB.

#### Concentration Study for CCL17 Antigen Detection.

Amperometric curves showed a reduction of oxidized TMB species, where consumption of reactants with time produced a constant concentration gradient and mass-transfer limited currents (Figure 3b). Immunosensors showed significant discrimination between control and sample mass-transfer limited currents at 120 s for spiked CCL17/TARC concentrations  $\geq 194$  pg/mL ( $p$ -value  $< 0.05$ ) (Figure 3c).



**Figure 4.** (a) Amperometric curves for functionalized electrodes following incubation with patient serum samples ( $n = 3$ ). (b) Electrode current responses at 120 s following incubation with patient serum samples ( $n = 3$ ). (c) Electrode current responses categorized into patient groups, based on known CCL17/TARC concentration; group A (1–5 ng/mL), group B (5–10 ng/mL), group C (10–50 ng/mL), and group D (>50 ng/mL). Asterisks indicate statistical significance, where  $p < 0.05$ ; cHL denotes classic Hodgkin lymphoma.

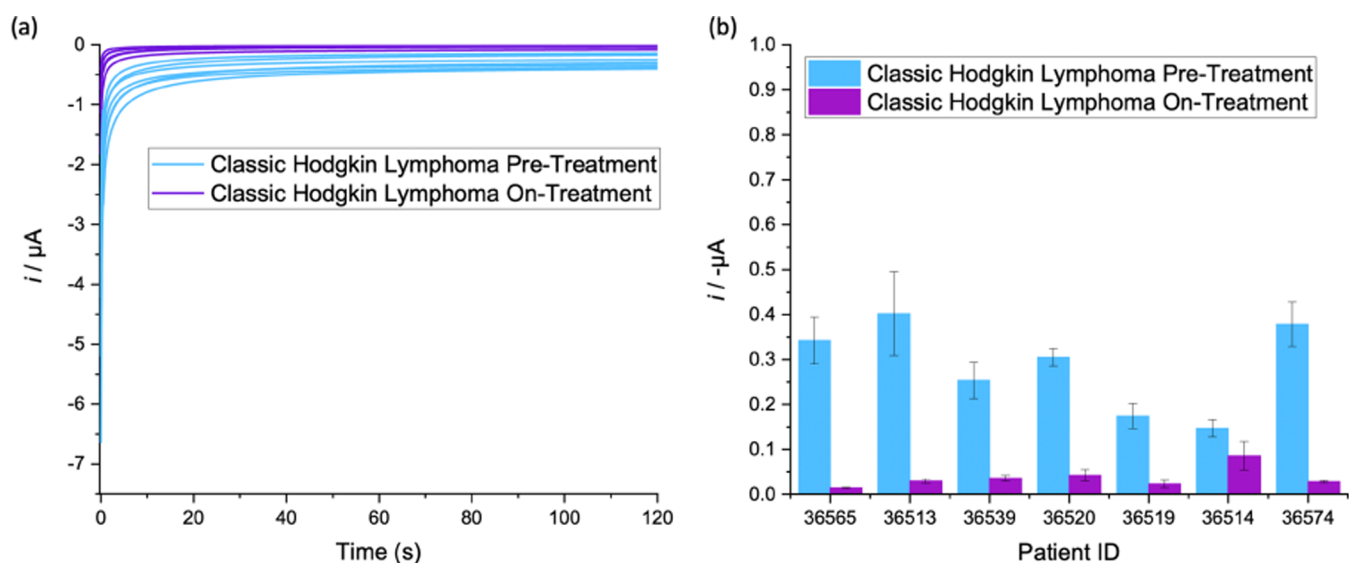
Signals were not linear over the specified concentration range, which is unsurprising given the wide concentration range. Mass-transfer limited currents at 120 s displayed a strong positive relationship with CCL17/TARC concentration,  $R^2 = 0.986$ , for concentrations statistically different from control measurements ( $p$ -value  $< 0.05$ ) (Figure 3d). 4PL regression was suitable for analysis as the model has been employed in electrochemical immunoassays<sup>49</sup> and better represents biological systems that display nonlinear behavior over wide concentration ranges. Interassay CV for mass-transfer limited currents at 120 s was 14.6% for all spiked CCL17/TARC concentrations (24–50 000 pg/mL). Additionally, interassay CV for mass-transfer limited currents at 120 s was 12.6% for CCL17/TARC concentrations (194–50 000 pg/mL) statistically significant from control samples ( $p$ -value  $< 0.05$ ). Lower and upper limits of quantitation were 387 and 50 000 pg/mL, respectively (based on  $CV \leq 20\%$ ), with a limit of detection of 194 pg/mL.

Comparatively, the biosensor demonstrates a superior dynamic range, with an upper limit of quantitation of 50 000 pg/mL CCL17/TARC compared to 2000 pg/mL for the equivalent colorimetric ELISA platform. The biosensor demonstrates reduced test sensitivity at 194 pg/mL when compared to the equivalent colorimetric ELISA test at 7 pg/mL.

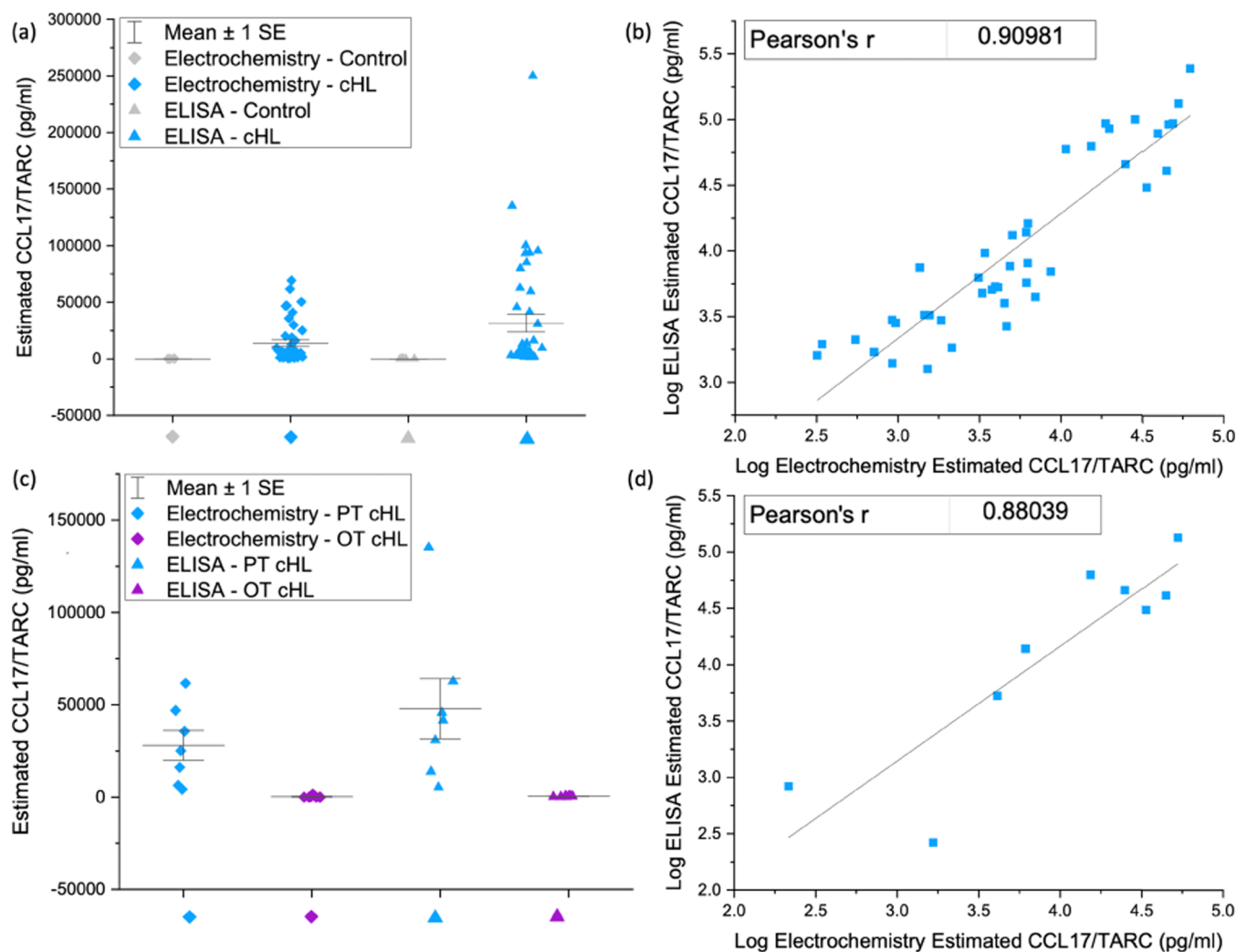
Nevertheless, test sensitivity is well below the proposed clinical cutoff for CCL17/TARC in cHL patients at 1000 pg/

mL<sup>50</sup> and therefore shows significant promise for clinical testing. However, the test showed reduced precision to quantify CCL17/TARC around the clinical cutoff, with unexpectedly raised signals for 775 pg/mL CCL17/TARC. Therefore, test precision and accuracy must improve during future assay development for patients that have CCL17/TARC concentrations near the clinical cutoff, although preliminary findings are extremely encouraging for clinical testing.

**Detection of CCL17/TARC in Pretreatment Patient Serum Samples.** Electrochemical immunosensors measured significant increases in currents at 120 s for all 42 cHL patients relative to healthy volunteers ( $p$ -value  $< 0.05$ ) (Figure 4a,b). Interassay CV of 19.5% across all patients was greater than previous concentration studies, likely attributable to the complexity of biologically rich serum derivatives. Nevertheless, the biosensor demonstrates the capacity to discriminate between healthy volunteers and cHL patients that have raised CCL17/TARC levels, which reflects ~90% of all cHL patients.<sup>1,7</sup> Electrode responses were significantly different between healthy volunteers and all cHL patient groups ( $p$ -value  $< 0.05$ ), stratified according to known CCL17/TARC concentrations (Figure 4c). Hence, the biosensor has the potential to quantify serum CCL17/TARC in cHL patients, which is important clinically as CCL17/TARC correlates with tumor burden.<sup>3–8</sup> However, further testing must be conducted on cHL patients with known CCL17/TARC levels at the



**Figure 5.** (a) Amperometric curves for paired patient serum samples tested at pretreatment and on-treatment clinical stages ( $n = 3$ ). (b) Plot of electrode current responses at 120 s for clinical patient serum samples ( $n = 3$ ).



**Figure 6.** (a) Plot of biosensor and ELISA test predictions for CCL17/TARC in pretreatment patients. (b) Correlation of predicted CCL17/TARC in pretreatment patients between the biosensor and ELISA tests. (c) Plot of the biosensor and ELISA test predictions for CCL17/TARC in pretreatment (PT) and on-treatment (OT) patient samples. (d) Correlation of predicted CCL17/TARC in pretreatment (PT) and on-treatment (OT) patient samples between the biosensor and ELISA tests.

clinical cutoff to confirm that the test identifies all cHL patients.

To the best of our knowledge, our findings demonstrate, for the first time, sensitive electrochemical detection of serum CCL17/TARC from pretreatment cHL patients. Electrochemical detection of CCL17/TARC has potential for rapid, economical point-of-care triage testing of cHL patients, following further assay optimization and utilization of printed electrodes. Clinically, the test would provide a minimally invasive and highly accessible platform to promote early detection of cHL and enable timely referral for diagnosis with lymph node biopsy. Practically, discriminatory signals can be generated in seconds to minutes and require minimal user interpretation, which offers the potential to streamline the diagnostic pathway. Economically, electrochemical biosensors offer a scalable technology with the adoption of printed electrodes, which would minimize test costs for healthcare. Presently, time-to-result from sample introduction is 2 h 42 min; thus, reduction in assay time is required to enable while-you-wait results. Strategies to streamline the test toward point-of-care include a reduction in or elimination of wash steps and reduction in assay incubation times. Incubation temperature, pH, and ionic strength of solutions should also be carefully considered, as these factors influence the equilibrium constant of antigen–antibody reactions. Secondary antibody concentration should also be optimized, as this influences the law of mass action governing antibody–antigen reactions. Direct conjugation of horseradish peroxidase to secondary antibodies prior to immobilization would further reduce time-to-result. Practically, whole blood testing should be considered to determine whether upfront sample processing can be eliminated. The development of instrumentation that integrates with the biosensor to enable automation of assay procedures should be strongly considered to streamline clinical workflows.

Biosensor reproducibility must be improved for clinical testing, given the current interassay CV of 19.5% for patient samples. Interassay CV  $\leq 15\%$  is acceptable for bioanalytical tests, although  $\leq 20\%$  is deemed acceptable for measurements approaching the lower limit of quantitation.<sup>51–53</sup> Test variability is likely attributable to variable SAM formation, detailed in reproducibility studies. Electrode topography is also likely partly responsible for the signal variation. SEM revealed a degree of surface roughness with small scratches on electrodes due to repeated mechanical polishing<sup>54</sup> (Figure S4). AFM confirmed superficial electrode scratches, although substrates were relatively smooth with good surface homogeneity (Figure S5). These observations are reassuring as high surface roughness promotes poor thiol organization and hinders SAM formation.<sup>55,56</sup> Furthermore, intra-assay CV for 23 patient samples was  $\leq 15\%$ , providing evidence the biosensor has potential for clinical testing.

**Evaluating Treatment Response through Serum CCL17/TARC Detection.** Testing was performed on cHL patient serum obtained pre- and on-treatment to determine biosensor utility for treatment response monitoring. Immunosensors measured significant decreases in current at 120 s between pre- and on-treatment groups ( $p$ -value  $< 0.05$ ), and for all paired cHL patient samples ( $p$ -value  $< 0.05$ ) (Figure 5a,b). Thus, the biosensor has the potential for monitoring treatment response, provided CCL17/TARC is clinically utilized in the future. Recently, decreases in CCL17/TARC in cHL patients have been shown to inform treatment response

after one chemotherapy cycle,<sup>5,25</sup> which would facilitate earlier identification of refractory patients, and address false-positive/negative results associated with interim PET/CT.<sup>8,23,24</sup> Likewise, CCL17/TARC has clinical potential to identify HL patients unresponsive to allogeneic stem cell transplants.<sup>5,7,57</sup> Thus, the biosensor represents a promising tool as an adjunct or alternative to interim PET-CT, with the potential to support development of personalized treatment strategies.

**Comparison of Electrochemical Immunosensor with CCL17/TARC ELISA Platform.** The concentration of serum CCL17/TARC determined using the electrochemical biosensor was significantly lower than the Human Quantikine CCL17/TARC colorimetric ELISA test ( $p$ -value  $< 0.05$ ) (Figure 6a). The biosensor underestimated CCL17/TARC in 33/42 patient samples and was particularly evident for patients with very high CCL17/TARC levels. Optimization of sample dilution should be considered to address poor test performance for patients with high CCL17/TARC levels. Nevertheless, biosensor quantification of CCL17/TARC in pretreatment patients positively correlated (Pearson's  $r = 0.910$ ) with the ELISA test (Figure 6b). The biosensor also underestimated serum CCL17/TARC for pre- and on-treatment cHL samples compared to the ELISA test (Figure 6c). However, measurements still positively correlated (Pearson's  $r = 0.880$ ) with the ELISA test (Figure 6d). The biosensor was unable to estimate serum CCL17/TARC for three healthy volunteers and five cHL patients during chemotherapy, which were all quantified by ELISA. Signals for these patients were below the lower interpolation limit of the calibration curve, and thus it was not possible to accurately quantify CCL17/TARC concentrations. However, serum CCL17/TARC concentration in these samples was in the low normal range, well below the proposed clinical cutoff for raised CCL17/TARC, and therefore would not affect the clinical usefulness of the assay.

Analytical discrepancies between the biosensor and ELISA test may be attributed to reduced sample volumes at 25  $\mu\text{L}$  for electrodes compared to 100  $\mu\text{L}$  for ELISA plates. Substrate solution volumes may contribute to analytical errors; 500  $\mu\text{L}$  is used for electrochemical tests compared to 100  $\mu\text{L}$  for ELISA, which may dilute signals. Primary antibody concentrations were greater for the biosensor and may introduce analytical error, since excessive concentrations can negatively influence antibody–antigen affinity through steric hindrance.<sup>58</sup> Thus, primary antibody titration experiments and a thorough evaluation of the assay protocol must be conducted in future studies to realize the clinical potential of the biosensor.

## CONCLUSIONS

The need for earlier diagnosis of cHL is well recognized and forms part of a wider key healthcare strategy to detect 75% of all cancers at an early stage in the U.K. by 2028.<sup>59</sup> An electrochemical biosensor for serum CCL17/TARC detection has the potential to facilitate triage of patients who have a differential diagnosis that includes cHL in primary care and has the potential to permit monitoring of chemotherapy response in secondary care. The electrochemical biosensor has demonstrated quantitative detection of CCL17/TARC with high sensitivity, linearity, and a large dynamic range (387–50 000  $\text{pg/mL}$ ). The biosensor demonstrated successful discrimination between serum samples of all cHL patients and healthy volunteers, which shows considerable promise for clinical translation of a point-of-care triage strategy. Additionally, the biosensor showed the ability to qualitatively measure



decreases in serum CCL17/TARC between all seven paired pre- and on-treatment cHL patient samples, which provides the potential to measure treatment response during chemotherapy. Overall, our preliminary findings have demonstrated considerable potential for electrochemical detection of serum CCL17/TARC in clinical samples and represent an important step toward the development of a rapid triage and treatment response test for cHL.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.1c00972>.

Supplementary methods; patient information (Table S1); electrochemical cleaning (Figure S1); SAM characterization: probability distribution (Figure S2); reproducibility study (Figure S3); and evaluation of electrode surfaces (Figures S4 and S5) (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

**Christopher Rinaldi** – WestCHEM, Department of Pure and Applied Chemistry, Technology and Innovation Centre, University of Strathclyde, Glasgow G1 1RD, U.K.;  
ORCID: [orcid.org/0000-0002-9055-7480](https://orcid.org/0000-0002-9055-7480); Phone: +44 (0) 141 444 7000; Email: [christopher.rinaldi@strath.ac.uk](mailto:christopher.rinaldi@strath.ac.uk)

### Authors

**Damion K. Corrigan** – Department of Biomedical Engineering, University of Strathclyde, Glasgow G1 1QE, U.K.

**Lynn Dennany** – WestCHEM, Department of Pure and Applied Chemistry, Technology and Innovation Centre, University of Strathclyde, Glasgow G1 1RD, U.K.;  
ORCID: [orcid.org/0000-0002-5481-1066](https://orcid.org/0000-0002-5481-1066)

**Ruth F. Jarrett** – MRC-University of Glasgow Centre for Virus Research, Glasgow G61 1QH, U.K.

**Annette Lake** – MRC-University of Glasgow Centre for Virus Research, Glasgow G61 1QH, U.K.

**Matthew J. Baker** – DXCOVER Ltd., University of Strathclyde, Technology and Innovation Centre, Glasgow G1 1RD, U.K.

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acssensors.1c00972>

### Author Contributions

<sup>†</sup>D.K.C., L.D., and M.J.B. contributed equally to this work. R.F.J., D.K.C., and M.J.B. conceived the study. C.R., D.K.C., and M.J.B. designed experimental methodology. C.R. performed experiments and analysis. L.D. provided instrumentation. M.J.B., D.K.C., and L.D. supervised the study. R.F.J. and A.L. provided patient samples and ELISA data. C.R. and R.F.J. wrote the manuscript.

### Notes

The authors declare no competing financial interest.

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