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Authorship contributions

NG designed and performed the research, analysed and interpreted data and wrote the manuscript. SS performed the research and wrote the manuscript. MR performed the statistical analysis. BH, JT, TB and RB collected data and performed research. BH, SIP, RJ, BRA and EAC performed research and interpreted data.

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NG reports consulting for Janssen, Pharmacyclics, Juno Therapeutics, Gilead, Kite Pharma, TG Therapeutics, Seattle Genetics, Spectrum, Celgene; speaker fees for Janssen, Gilead, AbbVie, Seattle Genetics; research funding from TG Therapeutics, Pharmacyclics, Seattle Genetics, Juno Therapeutics, and Forty Seven Inc. RJ reports consulting for Janssen, Pharmacyclics, TG Therapeutics, Gilead, AbbVie, Spectrum, Juno Therapeutics; speaker fees from Pharmacyclics, Genentech, Astra Zeneca; research funding from Pharmacyclics and TG Therapeutics.

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Supporting Information

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

Table SI. Dose modifications based on organ function.

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Analysis of human papillomavirus infection and leukaemic infiltrate in cutaneous squamous cell carcinoma from patients with chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia (CLL) patients have a greatly increased risk of developing cutaneous squamous cell carcinoma (cSCC), which can be particularly aggressive, as reflected by increased levels of recurrence and metastasis. Consequently, cSCC is a major contributor to the mortality of CLL patients (Brewer *et al*, 2014; Velez *et al*, 2014). New

Zealand (NZ) has one of the highest national rates of cSCC and we have previously reported that cSCC rates in CLL patients from a single NZ centre are three-fold higher again.

The increased incidence/aggressiveness of cSCC is thought to reflect, at least in part, immunosuppression associated with CLL (Brewer *et al*, 2014). Human papillomaviruses (HPV) have been implicated in cSCC development in individuals with immune dysfunction, and it has been hypothesised that cSCC from CLL patients may similarly harbour HPV. Although HPV has been detected in a proportion of cSCC from CLL patients, these studies analysed only small numbers of patients (≤9) (Flynn *et al*, 2010; Peretti *et al*, 2014; Hampras *et al*, 2018).

Several studies have reported that cSCC from CLL patients is associated with the presence of a leukaemic infiltrate (Smoller & Warnke, 1998; Mehrany *et al*, 2003; Wilson *et al*, 2010). The presence of immunosuppressive CLL cells potentially modulates anti-tumour immune responses and therefore, clinical progression. However, to date, only limited studies on these infiltrates have been performed and it is unclear whether the infiltrates are associated with particular tumour features (Smoller &

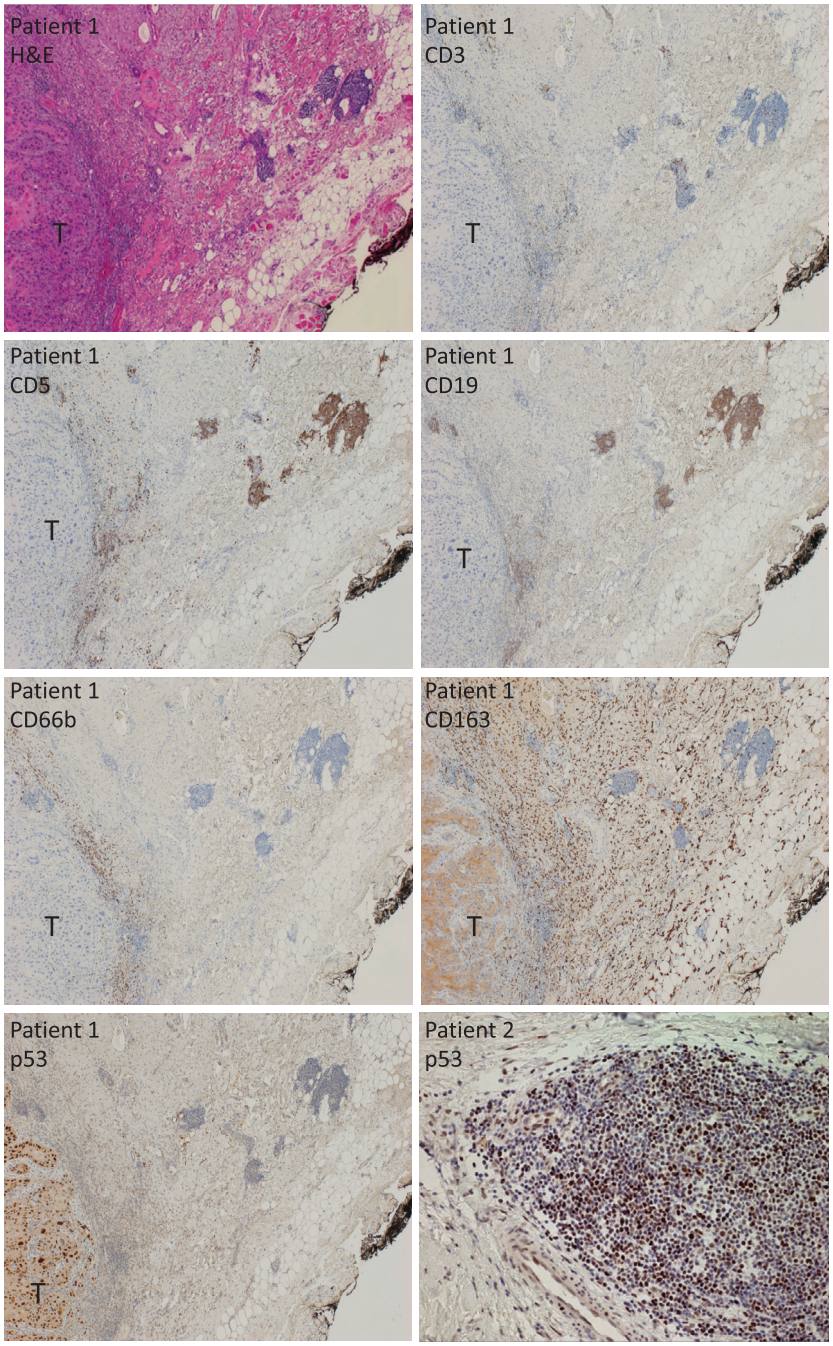

Fig 1. Images of representative cutaneous squamous cell carcinoma with a leukaemic infiltrate. Patient 1: Low power (×50) images of immuno-histochemical staining of Patient 1, demonstrating the location of the leukaemic infiltrate (CD5+, CD19+) in relation to the tumour (T) and other leucocyte populations. Strong p53 staining is seen within the tumour. Patient 2: High power view (×200) of Patient 2, demonstrating the expression of p53 protein by some leukaemic cells. Monoclonal antibodies used for immuno-histochemistry were CD19 (1:200, Clone LE-CD19), CD5 (1:50, Clone 4C7), CD3 (1:40, Clone F7.2.38), CD163 (1:50, Clone 10D6) and p53 (1:20, Clone DO-1) from ThermoFisher Scientific (Rockford, IL, USA). CD66b (1:1000, Clone G10F5) was from BD Biosciences. Specificity of staining was confirmed using a Mouse IgG1 isotype control (1:200, Clone MOPC21) from Sigma-Aldrich (St. Louis, MO, USA). H&E, haematoxylin and eosin.

Warnke, 1998; Mehrany *et al*, 2003; Wilson *et al*, 2010). The interaction of CLL cells with immune components, particularly macrophages and T cells, is known to be important for both CLL survival and the development of an immune-subversive milieu (Burger & Gribben, 2014). However, the localization of leukaemic infiltrates in relation to other cell types is unknown.

The presence of HPV and/or leukaemic infiltrate was therefore analysed in cSCC tissue excised from 27 CLL patients.

Archived cSCC tissue (formalin-fixed paraffin-embedded, FFPE) from CLL patients was obtained with ethical approval (16/STH/75/AM02). Immunohistochemistry was performed following antigen retrieval (Tris-EDTA, pH9) using primary monoclonal antibodies and an Envision+ Dual Link System-horseradish peroxidase 3,3′-Diaminobenzidine-positive (K4065; Dako, Glostrup, Denmark), following the manufacturer's protocols.

DNA was extracted from tissue using the The QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. HPV presence was detected using an nCounter™ Custom CodeSet (NanoString Technologies, Inc., Seattle, WA, USA) designed to detect the E6 and L1 genes of 12 viral strains of interest, in addition to four reference genes.

Clusters of lymphocytic infiltrate expressing both CD19 and CD5 were observed in 16/27 samples. These clusters varied in size and number, with 7/16 having <4 clusters visible in the section. There were no intra-tumoural leukaemic clusters observed, but in 11/16 samples, clusters were observed close to the tumour margin. Leukaemic clusters were observed in the subcutaneous fat and/or dermis in 14/16 samples. A feature of the CLL microenvironment in lymphoid tissue is the intertwining of CD163+ nurse cells with leukaemic cells, which is thought to promote CLL survival. High numbers of CD163+ macrophages were observed throughout all samples. However, relatively few were observed within the leukaemic infiltrate.

Granulocytes and T cells are also thought to be important contributors to the CLL microenvironment (Burger & Gribben, 2014). Only low number of CD66b+ granulocytes were observed, and none were found within the infiltrate. In 14/16 samples CD3+ cells were observed as a minor population in the leukaemic infiltrate.

Table I. Patient and tumour characteristics of samples with or without leukaemic infiltrate.

	CLL Patient grouping		
		Leukaemic infiltrate in skin*	
Characteristic	All	Yes	No
Patient number	27	16	11
Age (years)			
Median	72	72	72
Range	54–92	60–92	54–83
Median OS, years (95% CI)	6·0 (5·3–8·6)	6·1 (5·3–9·3)	4·8 (3·1–9·6)
Time from CLL diagnosis to SCC analysis, years; median (95% CI)	3·8 (3·7–6·9)	5·2 (3·7–7·8)	3·8 (1·7–7·8)
Total SCC > 2 (%)	48	44	55
Male (%)	89	94	82
Binet stage B, C (%)	56	50	64
HPV+ (%)	30	25	36
Poorly differentiated (%)	19	20	18
Tumour diameter, mm; median (range)	20·0 (3–55)	15·0 (3–28)	25 (7–55)†
Tumour size > 20 mm (%)	41	31	55
HPV types detected (*n*)			
Nil	19	12	7
5	1	1	0
5, 20	1	0	1
24	1	0	1
24, 8	2	1	1
24, 8, 20	1	1	0
24, 8, 38	1	0	1
24, 15, 38	1	1	0

Primary antibodies used to detect CLL infiltrate were CD19 (1:200, Clone LE-CD19) and CD5 (1:50, Clone 4C7) from ThermoFisher Scientific (Rockford, IL, USA).

CI, confidence interval; CLL, chronic lymphocytic leukaemia; HPV, human papillomavirus; OS, overall survival; SCC, squamous cell carcinoma.

*CLL infiltrate was defined by CD19+, CD5+ staining of the same areas in sequential sections.

†$P = 0{\cdot}038$. Data were analysed using unpaired *t* test.

All cSCC were positive for p53 (also termed TP53), with strong expression in 13/27 samples. Expression of p53 by the leukaemic cells was observed in 4/16 samples.

Figure 1 shows representative images of cSSC with a leukaemic infiltrate.

Samples with and without leukaemic infiltrate were compared with respect to tumour and patient characteristics (Table I). There was no significant difference between these groups with regard to the age, sex, Binet stage or overall survival of the respective patients. However, there was a significant difference in tumour size between the groups ($P = 0{\cdot}038$).

Leukaemic infiltrates were found in the skin of 59% of CLL patients with cSCC. However, apart from small numbers of T cells, these infiltrates were not associated with other immune populations. This, together with their presence in the dermis and subcutaneous fat, suggests they are not part of a reactive process.

Previous studies investigating the HPV status of cSCC from CLL patients reported differing findings using polymerase chain reaction (PCR)-based methodologies: (i) 5/8 samples were HPV+ (Flynn *et al*, 2010); (ii) 1/7 samples expressed HPV17 (Peretti *et al*, 2014) and (iii) 3/9 samples were positive and expressed either HPV38, HPV15 or HPγV6 (Hampras *et al*, 2018). The current study targeted 12 β-HPV strains previously reported as being expressed in cSCC (HPV5, 8, 15, 16, 17, 20, 23, 24, 36, 38, 65, 197). A total of six of these HPV types were detected in 8/27 samples. In six of the HPV+ samples, 2–3 different HPV types were detected, with HPV24 present in five of these samples and HPV8 in four (Table I).

These HPV types were not detected in previous CLL studies. This probably reflects methodological differences, as the current study utilizes NanoString as opposed to PCR-based approaches. NanoString is better able to overcome the issues of low nucleic-acid yield and high sample degradation associated with FFPE tissue. In addition, unlike PCR, which has been used in other HPV studies, NanoString does not use any amplification steps that might introduce bias.

Larger studies have been performed using cSCC from immunocompetent individuals and a recent meta-analysis of those results reported that the β-HPV types detected in the current study were significantly associated with cSCC development (Chahoud *et al*, 2016). The exact biological relevance of β-HPV in cSCC remains unclear, but it is postulated that they play a role in the initiation of carcinogenesis. Therefore, the presence of HPV, even at low levels, in a substantial proportion of the cSCC from CLL patients suggests it may play a role in the development of these skin cancers.

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Author contributions

LP, RP and JM performed the research. SM, BH, JM and RP designed the study. JM, JG and RP analysed the data. BH, JM, RP and SM wrote the manuscript.

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Leucocytosis-induced plasma hyperkalaemia in samples conveyed by a pneumatic transport system: tips and tricks

In previous articles, including the recent case report published by Racek *et al* (2019), spurious hyperkalaemia has been documented in uncentrifuged plasma samples conveyed to laboratory by means of a pneumatic transport system (PTS). We also recently described the case of a child with high leucocytosis (i.e., 800×10^9/l), whose uncentrifuged plasma samples were transported to the laboratory by PTS and then revealed a spurious increase of plasma potassium and an unexpected artefactual decrease of sodium in plasma (Grzych *et al*, 2019). Given that these electrolyte abnormalities are most likely to be attributable to the use of PTS, it was suggested that PTS should be avoided for convening blood samples with leucocytosis (Racek *et al*, 2019). Nevertheless, avoidance of sample transportation with PTS did not apparently solved the problem in our previously published case (Grzych *et al*, 2019). On the other hand, although potassium measurement with a point of care (POC) device was found to considerably limit such interference - thus enabling a more reliable measurement – the use of POC testing is sometimes challenged by lack of resources or equipment. Therefore, in our earlier case report we hypothesized that the use of clot activator-based serum tubes could be seen as a reliable solution for preventing interference on potassium measurement in the plasma of patients with very high leucocytosis.

To validate this hypothesis, we retrospectively analysed the results of patients that showed spurious plasma hyperkalaemia related to leucocytosis and PTS usage at our local institution. The study population consisted of patients undergoing routine laboratory testing at Lille University Hospital. Patients received clear information that clinical data and/or residual blood samples could be used for research purposes. All information was retrieved from the human biological database, previously cleared by the French Ministry of Research (No. DC-2008-642), so that no written informed consent was necessary. Exclusion criteria were spurious hyperkalaemia due to haemolysis (i.e., haemolysis index >100), EDTA contamination (as revealed by abnormally low calcium concentration associated with high potassium values), blood drawn from (or above) intravenous lines (as reflected by extremely low or high values of infused analytes, such as sodium and glucose). We also exclude samples with >2 h delay between sample collection and centrifugation (Meng & Wagar, 2015). Patients with platelet counts $>500 \times 10^9$/l were also excluded, because thrombocytosis is another well-known cause of spurious hyperkalaemia (Ku *et al*, 2014). The analysis was hence limited to samples simultaneously drawn in a lithium-heparin tube without gel (4 ml BD Vacutainer™ Heparinized Tube [HT]; Becton Dickinson, Franklin Lakes, NJ, USA) and in a clotting activator blood tube without gel (4 ml BD Vacutainer™ Clot Activator Tubes [CAT]; Becton Dickinson), displaying both very high leucocyte count (i.e., $>100 \times 10^9$/l) and high plasma potassium concentration (i.e., >5·0 mmol/l). All blood tubes were transported to the local laboratory by PTS (Swisslog, Buchs, Switzerland). Briefly, tubes were first inserted into a disposable plastic bag, then placed in a second bag with parachutes propelled in a one-way air-flow system with speed limited to 6 m/s and soft-landing capabilities. Plasma and serum samples were immediately centrifuged at 4000 ***g*** for 5 min within 30 min of collection.

The final study population consisted of 10 patients, all with chronic lymphocytic leukaemia (CLL) and a median leucocyte count of 180×10^9/l (Table I). The median potassium value was 7·75 mmol/l in plasma and 4·35 mmol/l in serum, respectively (median difference, 2·8 mmol/l), and this difference was found to be highly statistically significant (Wilcoxon's paired-test, $P < 0{\cdot}001$) (Fig 1).

Several lines of evidence now suggest that potassium values can be sometimes unreliable in plasma samples with extreme leucocytosis that are conveyed to the laboratory by means of PTS (Plebani & Zaninotto, 2011). Our results suggest that this bias could be at least partly reduced by using serum, and is probably due to the fact that the rapid generation of a blood clot would at least partially prevent leucocyte injury during PTS transportation. The results of this retrospective analysis