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ORIGINAL ARTICLE

Dicloxacillin is an inducer of intestinal P-glycoprotein but neither dicloxacillin nor flucloxacillin increases the risk of stroke/systemic embolism in direct oral anticoagulant users

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Abstract

Aim: We aimed to assess if dicloxacillin/flucloxacillin reduces the therapeutic efficacy of direct oral anticoagulants (DOACs) and the underlying molecular mechanism.

Methods: In a randomized, crossover study, we assessed whether dicloxacillin reduces oral absorption of drugs through P-glycoprotein (P-gp) during 10 and 28 days of treatment. To study the impact of dicloxacillin/flucloxacillin on intestinal and hepatic expression of P-gp in vitro, we usd LS174T cells and 3D spheroids of primary human hepatocytes. Finally, we used nationwide Danish health registries and the UK's Clinical Practice Research Datalink to estimate hazard ratios (HRs) for the risk of stroke and systemic embolism following dicloxacillin/flucloxacillin exposure among DOAC users, using phenoxymethylpenicillin and amoxicillin as active comparators.

Results: Dicloxacillin reduced the area under the curve of dabigatran to a geometric mean ratio 10 days of 0.67 (95% confidence interval [CI]: 0.42–1.1) and geometric mean ratio 28 days of 0.72 (95% CI: 0.39–1.4), suggesting reduced oral absorption via increased P-gp expression. In vitro, dicloxacillin raised P-gp expression in both intestinal and liver cells, while flucloxacillin only affected liver cells. In the pharmacoepidemiologic study, dicloxacillin and flucloxacillin were not associated with increased risk of stroke/systemic embolism (dicloxacillin *vs*. phenoxymethylpenicillin HR: 0.93, 95% CI: 0.72–1.2; flucloxacillin *vs*. amoxicillin HR: 0.89, 95% CI: 0.51–1.5).

Conclusions: Dicloxacillin increases expression of intestinal P-gp, leading to reduced oral absorption of dabigatran. However, concomitant use of dicloxacillin/flucloxacillin

The authors confirm that the Principal Investigator for this paper is Ann-Cathrine Dalgård Dunvald and that she had direct clinical responsibility for patients.

was not associated with stroke and systemic embolism among DOAC users, suggesting no clinical impact from the drug–drug interaction between dicloxacillin/flucloxacillin and DOACs.

KEYWORDS
antibiotics, direct oral anticoagulants, drug–drug interactions, P-glycoprotein transporter, stroke, systemic embolism

1 | INTRODUCTION

P-glycoprotein (P-gp) is an essential drug transporter responsible for extruding toxins and xenobiotics out of cells and is key in limiting oral absorption of many drugs.[1,2] Therefore, drug–drug interactions through P-gp can potentially change the clinical safety and efficacy of many drugs.[3] Drug–drug interactions are particularly important for drugs with narrow therapeutic ranges, such as anticoagulants. We have previously shown that dicloxacillin and flucloxacillin, 2 commonly used antibiotics,[4] decreased the international normalized ratio in users of the vitamin-K antagonist warfarin[5,6] and, consequently, increased the risk of stroke and systemic embolism.[7] Dicloxacillin is known to induce **cytochrome P450 (CYP) 3A4**, **CYP2C9** and **CYP2C19**, while flucloxacillin is known to induce CYP3A4.[8,9] The drug–drug interaction between warfarin and dicloxacillin/flucloxacillin is, therefore, likely to be caused by induction of the drug-metabolizing enzymes involved in warfarin metabolism by dicloxacillin and flucloxacillin.[8,9] The Food and Drug Administration and the European Medicines Agency recommend investigating perpetrator drugs capable of inducing CYP3A4 for P-gp induction.[10,11] It is known that both dicloxacillin and flucloxacillin increase the activity of **pregnane X receptor (PXR)**, thereby inducing CYP3A4 and potentially also P-gp.[9] However, induction of P-gp has never been assessed for dicloxacillin or flucloxacillin.

Direct oral anticoagulants (DOACs), dabigatran etexilate, rivaroxaban, apixaban and edoxaban, are used to prevent stroke and systemic embolism in patients with nonvalvular atrial fibrillation (NVAF). DOACs are considered as safe and effective as warfarin in preventing stroke in patients with NVAF.[12] All 4 DOACs are substrates for P-gp, and apixaban, rivaroxaban and edoxaban are also substrates for CYP3A4.[13] With the increasing number of patients with atrial fibrillation (AF),[14] and a corresponding increase in DOAC use,[15] it is essential to understand potential drug–drug interactions with DOACs. Inducers of P-gp and/or CYP3A4 may decrease the plasma concentration of DOACs, potentially reducing the therapeutic efficacy and increase the risk of stroke or systemic embolism in patients with NVAF.

We aimed to assess the potential drug–drug interaction between dicloxacillin/flucloxacillin and DOACs using a translational approach. First, to understand whether dicloxacillin increases expression of P-gp and reduces oral absorption of drugs in healthy adults, we conducted a randomized, crossover clinical pharmacokinetic study to assess the effect of short- and long-term treatment with dicloxacillin. Secondly, we studied if dicloxacillin and flucloxacillin increase the expression of P-gp in vitro using intestinal and liver cells. Finally, we identified a cohort of real-world NVAF patients taking DOACs and investigated if short-term use of dicloxacillin or flucloxacillin was associated with an increased risk of stroke or systemic embolism.

What is already know about this subject

- Dicloxacillin is an agonist to the pregnane X receptor, which regulates CYP3A4 and P-glycoprotein. We previously showed that dicloxacillin induces CYP3A4.

What this study adds

- Dicloxacillin increased the intestinal expression of P-glycoprotein in LS174T cells and reduced the oral absorption of dabigatran in healthy adults. However, short-term use of dicloxacillin did not increase the risk of stroke/systemic embolism when coadministered with direct oral anticoagulants.

2 | METHODS

The methods used for the 3 components of this translational study, i.e., the clinical trial, the in vitro studies and the pharmacoepidemiologic studies, are summarized below. More detailed descriptions can be found in the Supplementary Material and Supplementary Codes, with descriptions of study designs and study populations, cell cultivation, laboratory analysis, data analysis and data sources for the pharmacoepidemiologic study.

2.1 | Clinical study

2.1.1 | Study design

We performed a randomized, unblinded, 2-period, crossover, clinical pharmacokinetic study (Figure 1). Twelve healthy adults were included

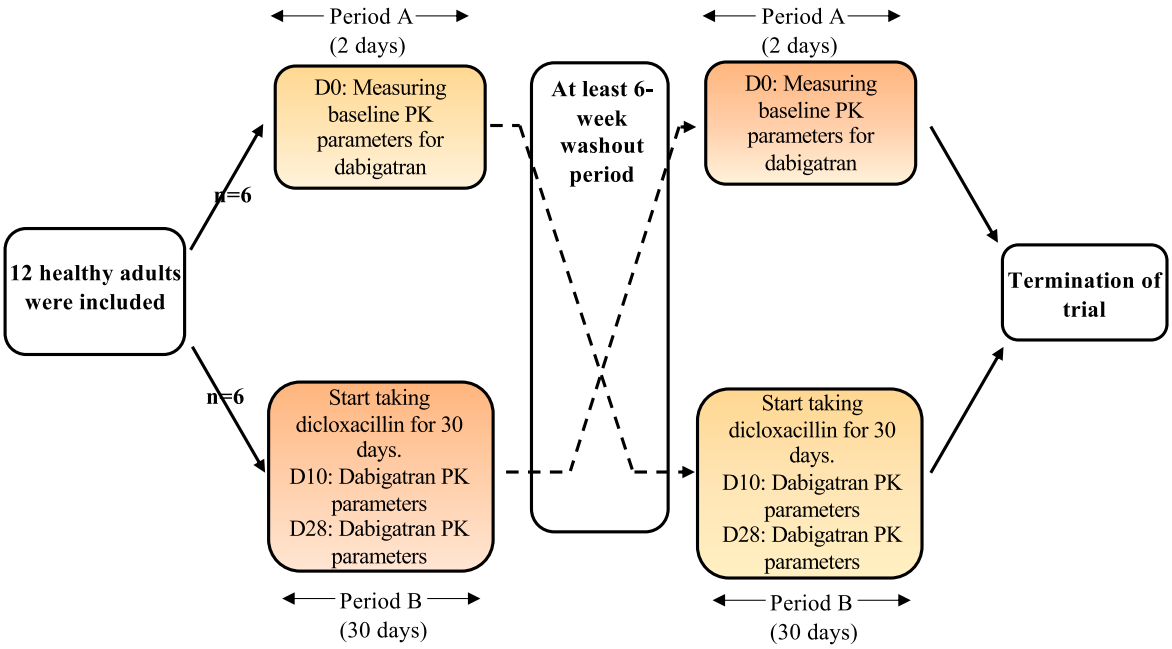

FIGURE 1 An overview of the clinical study design. D, day; PK, pharmacokinetic.

in the trial. We used dabigatran etexilate not because it was a DOAC but rather as a regulatory probe for P-gp activity. Our primary endpoint was change in area under the curve (AUC) of dabigatran after 28 days of dicloxacillin treatment compared to baseline. The secondary endpoints were changes in the full pharmacokinetic parameters of dabigatran etexilate and dabigatran after 10 and 28 days of dicloxacillin treatment compared to baseline. Healthy adults completed 2 periods, A and B, separated by a 6-week wash-out period (Figure 1). In period A, they ingested 150 mg dabigatran etexilate without concomitant administration of dicloxacillin. In period B, dicloxacillin was self-administered as 1 g 3 times daily for 30 days. On days 10 and 28, healthy adults ingested 150 mg of dabigatran etexilate. Randomization was carried out by a data manager using random.org. The list was uploaded in REDCap, which enabled investigators to randomize included healthy adults.

2.1.2 | Study medication

Healthy adults took 2500 mg dicloxacillin capsules (Dicillin, Sandoz, Copenhagen, Denmark) thrice daily for 30 days. They were instructed to take dicloxacillin at least 1 h before or 2 h after a meal. The Danish Physicians' Desk Reference recommends avoiding taking dicloxacillin with food for the treatment of *Staphylococcus aureus* in Denmark (www.pro.medicn.dk). Dabigatran etexilate (Pradaxa, 150-mg capsules, Boehringer Ingelheim, Copenhagen, Denmark) was administered at the trial location in the morning on the trial day in period A and on trial days 10 and 28 (period B). On trial days in period B, healthy adults were instructed to ingest dicloxacillin in the evening and skip the morning and afternoon doses.

2.1.3 | Sampling time

Blood samples were drawn before continuous administration of dicloxacillin (period A) and after 10 and 28 days of dicloxacillin treatment (period B; Figure 1). On each day, blood samples were drawn at baseline before drug administration and until 32 h after administration of dabigatran etexilate. Urine was collected at intervals from 0 to 32 h.

2.1.4 | Study approval

The clinical trial was conducted in accordance with the Helsinki Declaration and Good Clinical Practice and monitored by the Good Clinical Practice Unit, Odense University Hospital, Odense, Denmark. The study protocol was approved by the Danish Medicines Agency (identifier 2 021 082 148) and the Regional Scientific Ethical Committee of Southern Denmark (identifier S-20210118) and registered in the EudraCT database (identifier 2021–003814-37). The trial was registered at http://www.clinicaltrials.gov (identifier NCT05073627). All healthy adults consented to participate in the study. One author had full access to all the data in the study and took responsibility for its integrity and the data analysis.

2.1.5 | Laboratory determination of biological material

The concentration of dabigatran and dabigatran etexilate in plasma and urine samples was determined by liquid chromatography–tandem mass spectrometry.

2.1.6 | Statistical and pharmacokinetic analysis

A sample size of 10 individuals was needed to detect a difference of ≥40% of dabigatran AUC with a power of 80%, and a 2-sided significance level of 5%. To conduct the statistical analysis, we used the previously described method.[16] We calculated demographic data with median and interquartile ranges (IQRs) and ranges. We calculated pharmacokinetic endpoints with noncompartmental analysis and presented them as medians with IQR and geometric mean ratios (GMRs) with 95% confidence intervals (CIs).

2.2 | In vitro study

2.2.1 | LS174T cells

We treated LS174T cells (acquired from American Type Culture Collection [CL-188]) with either dicloxacillin or flucloxacillin (concentration range 1–500 μM) for 72 h. Induction of P-gp (*ABCB1*) mRNA was measured relative to a 0.1% dimethyl sulfoxide control. We conducted 3 experiments for the mRNA analysis. The induction parameters maximal effect (E_{max}) and the half-maximal concentration (EC_{50}) were derived for P-gp (*ABCB1*) when induction was present.

2.2.2 | 3D spheroid of primary human hepatocytes

We treated 3D spheroid of primary human hepatocytes (PHH) with either dicloxacillin or flucloxacillin (concentration range 0.150–250 μM) for 96 h. To explore novel drug–drug interactions, we also explored regulation of other drug transporters than P-gp by dicloxacillin and flucloxacillin. We used 3 donors for all mRNA analysis and 2 donors for protein analysis. Only 1 donor had detectable expression of P-gp and **multidrug resistance-associated protein 2** (**MRP2**), while organic cation transporter 1 (OCT1) was analysed in 1 experiment. Induction of transporter/enzyme mRNA was measured for P-gp (*ABCB1*), **breast cancer resistance protein** (**ABCG2, BCRP**), **carboxylesterase 1** (***CES1***), MRP2 (*ABCC2*) and **organic anion transporting polypeptide 1B1** (***SLCO1B1***, **OATP1B1**). Induction of transporter protein was measured for P-gp, MRP2, OCT1, sodium-taurocholate cotransporting polypeptide and MRP6. The induction parameters E_{max} and EC_{50} were derived for each gene and protein when induction was present.

2.3 | Pharmacoepidemiologic study

Using Danish registries, we identified all DOAC users from 2011–2022 and compared the risk of stroke and systemic embolism between users of dicloxacillin and, as an active comparator, phenoxymethylpenicillin. We conducted the same study using data from the UK's Clinical Practice Research Datalink (CPRD) and compared flucloxacillin users with the active comparator amoxicillin since phenoxymethylpenicillin is not commonly used in the UK. In both cohorts, we also compared dicloxacillin/flucloxacillin users with users not taking antibiotics. Phenoxymethylpenicillin and amoxicillin were selected as active comparators as they are known not to affect PXR.[17] PXR regulates the expression of CYP3A4 and P-gp,[18] and, therefore, we do not expect CYP3A4 or P-gp to be affected by phenoxymethylpenicillin or amoxicillin.

2.3.1 | Study population

Data was analysed as a cohort and a case-crossover study. Patients ≥18 years were included in the cohort when they received their first-ever prescription of DOACs (using a 2-year look-back period) and if they had a prescription of ≤200 capsules of dicloxacillin/flucloxacillin. The time for prescription fills for dicloxacillin/flucloxacillin or phenoxymethylpenicillin/amoxicillin was set as the index date. Nonusers of antibiotics (defined as having filled no antibiotic prescription within 30 days before the start of follow-up) were assigned a random index date during DOAC use. In 1 analysis, we matched patients receiving dicloxacillin/flucloxacillin to patients receiving phenoxymethylpenicillin/amoxicillin and, in the second analysis, patients receiving dicloxacillin/flucloxacillin were matched to patients receiving no antibiotic treatment, using propensity scores. We evaluated the covariates at the index date, applied a 180-day look-back period to evaluate prescription data, and used all available data ever for diagnoses. Propensity score models included age, sex, calendar year, season, time since first cohort entry (years), $CHAD_2DS_2$-VASc-score, HAS-BLED-score, comorbidities, Charlson comorbidity index, concomitant use of drugs that potentially interacts with DOACs, and drugs acting as markers of cardiovascular disease. At the index date, patients were followed from day 5 until day 20 (follow-up window; Figure S1), and only the first outcome was included in the analysis. We excluded the first 5 days after prescription fill of antibiotics, based on a former study investigating the time it takes for induction of intestinal CYP3A4 to occur.[19] Since PXR is involved in regulation of both CYP3A4 and P-gp, we believe that 5 days should be sufficient to allow induction of P-gp, in line with induction of CYP enzymes.[18]

2.3.2 | Cohort analysis

We estimated the 20-day risk of stroke or systemic embolism with a 95% CI for all 4 DOACs in each exposure group using Poisson regression. We also used a Cox regression to calculate the hazard ratio (HR) and 95% CI for stroke and systemic embolism associated with dicloxacillin/flucloxacillin *vs.* phenoxymethylpenicillin/amoxicillin and *vs.* no antibiotic use. We also evaluated the outcome incidence rate per 1000 person-years for dicloxacillin/flucloxacillin, phenoxymethylpenicillin/amoxicillin and no use of antibiotic.

2.3.3 | Sensitivity analysis

In sensitivity analyses, we performed stratified analysis by age, sex and individual DOACs. Furthermore, we excluded patients with a history of diabetes, prior use of dicloxacillin/flucloxacillin, hospitalization within 10 days prior to index date (only in Danish data since this information was not available from CPRD), and use of other antibiotics within 30 days prior to the index date. We also estimated the risk of bleeding and if the indication for DOAC treatment had any influence (including patients with deep vein thrombosis and pulmonary embolism), setting the outcome to new venous thromboembolism. Lastly, we extended the risk window from 5–20 days to 5–30 days to estimate if delayed outcomes are present after dicloxacillin/flucloxacillin treatment.

2.3.4 | Case crossover analysis

As a supplementary analytic approach, we deployed a case-crossover-design,[20] a self-controlled design restricting to those experiencing stroke and systemic embolism, investigating whether this happened in timely relation to their use of dicloxacillin/flucloxacillin. Patients experiencing an outcome served as their own control and contributed with data for both the exposed and unexposed follow-up time (Figure S2).

2.4 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.[21]

3 | RESULTS

3.1 | Clinical trial

We recruited 12 healthy adults from January 2022 to June 2022. Ten healthy adults (4 women and 6 men) completed the study according to the protocol, and we reached the predetermined sample size of 10. Two did not complete the study due to adverse events. The median age was 24 years (IQR; 23–25; range 22–35 years), and the median body mass index was 23.3 kg m^{-2} (IQR; 22.3–26.8; range 20.4–27.5 kg m^{-2}). Following dicloxacillin treatment, adverse events were reported among the 12 healthy adults: diarrhoea ($n = 7$), discomfort ($n = 3$), stomach pain ($n = 2$), vaginal yeast infection ($n = 2$), pain in the oesophagus ($n = 1$), heartburn ($n = 1$), dyspepsia ($n = 1$), constipation ($n = 1$), nausea ($n = 1$) and urinary tract infection ($n = 1$). No adverse events were reported following a single oral dose of dabigatran etexilate. All were described in the Summary of Product Characteristic for dicloxacillin, except for heartburn, constipation and urinary tract infection. None of the adverse events were deemed serious. Two healthy adults withdrew from the study. Of the 10 participants who completed the study, 5 started in period A and 5 started in period B. Only data on drug concentration from the 10 individuals who completed the whole study are included in the analysis. However, due to very low concentrations, it was not possible to detect dabigatran etexilate in urine samples from healthy adults.

The $AUC_{0\text{-inf}}$ of dabigatran were reduced after 10 and 28 days of treatment with dicloxacillin to a GMR of 0.67 (CI 95%: 0.42–1.1) and GMR of 0.72 (95% CI; 0.39–1.4), respectively. The maximum concentration (C_{max}) of dabigatran were also reduced (GMR 10 days of 0.69 (95% CI: 0.40–1.2) and GMR 28 days of 0.71 (95% CI: 0.31–1.7; Figure 2, Table 1)). Individual data of C_{max} for dabigatran show considerable interindividual variability before and after 10 and

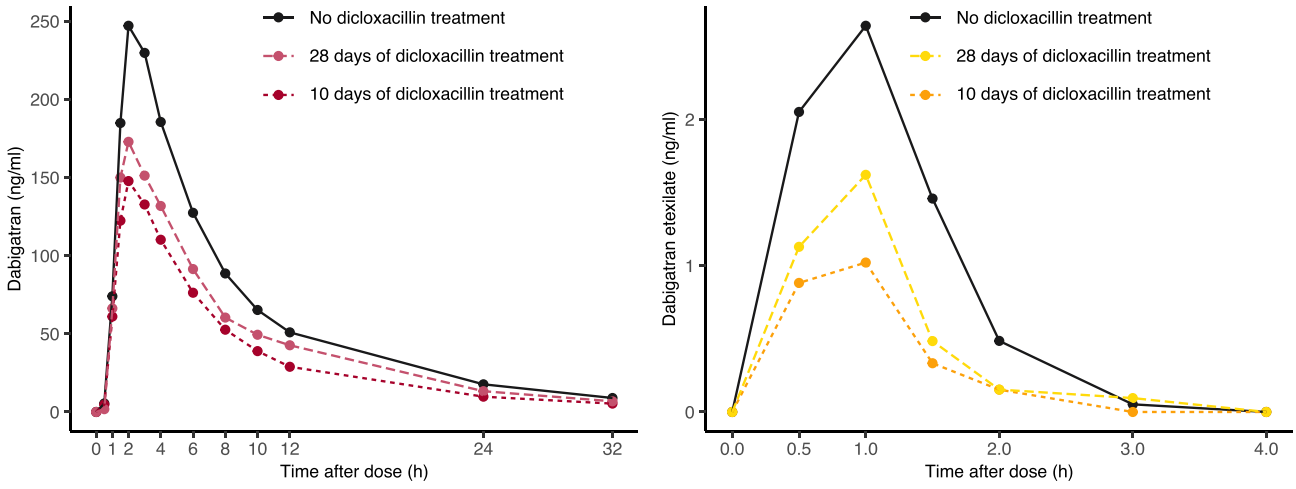

FIGURE 2 Mean plasma concentration–time curve for dabigatran and the prodrug dabigatran etexilate after 10 and 28 days of dicloxacillin treatment in 10 healthy adults. Both curves show signs of induction of P-gp.

TABLE 1 Noncompartmental analysis from the clinical trial of dabigatran and dabigatran etexilate shows alterations in pharmacokinetic parameters of dabigatran and dabigatran etexilate after 10 and 28 days of treatment with 1 g dicloxacillin thrice daily.

Drug	Parameters	Baseline, median (IQR)	After 10 days of dicloxacillin, median (IQR)	GMR 10 days (95% CI)	After 28 days of dicloxacillin, median (IQR)	GMR 28 days (95% CI)
Dabigatran	$AUC_{0\text{-inf}}$ (ng h mL^{-1})	1540 (796–2236)	1105 (630–1530)	0.67 (0.42–1.1)	1133 (897–1745)	0.72 (0.39–1.4)
	C_{max} (ng mL^{-1})	193 (95–357)	164 (70–212)	0.69 (0.40–1.2)	186 (124–233)	0.71 (0.31–1.7)
	T_{max} (h)	2.0 (2.0–2.0)	2.0 (2.0–2.0)	0.92 (0.75–1.1)	2.0 (1.5–2.0)	0.90 (0.73–1.1)
	$T_{½}$ (h)	7.5 (7.2–9.4)	8.0 (7.4–8.5)	0.95 (0.80–1.1)	7.5 (7.0–7.8)	0.99 (0.72–1.4)
	CL/F (l h^{-1})	106 (67–193)	138 (98–238)	1.5 (0.95–2.4)	137 (86–167)	1.4 (0.74–2.6)
	CL_R (l h^{-1})	6.8 (6.2–8.2)	6.7 (6.4–7.8)	1.0 (0.96–1.1)	6.6 (5.9–7.9)	1.0 (0.86–1.2)
	[a]Ae (mg)	11 (4.6–18)	6.3 (4.0–11)	0.68 (0.43–1.1)	8.6 (5.2–15)	0.70 (0.34–1.4)
[b]Dabigatran etexilate (P-gp substrate)	$AUC_{0\text{-inf}}$ (ng h mL^{-1})	2.6 (1.2–4.1)	0.45 (0.24–1.1)	0.32 (0.13–0.79)	1.3 (0.35–2.7)	0.62 (0.17–2.3)
	C_{max} (ng mL^{-1})	4.1 (1.9–6.3)	1.2 (0.90–1.8)	0.43 (0.23–0.82)	2.0 (1.3–2.8)	0.78 (0.35–1.8)
	T_{max} (h)	0.99 (0.62–1.0)	1.0 (0.62–1.0)	0.98 (0.57–1.7)	1.0 (0.98–1.0)	0.92 (0.68–1.2)

Abbreviations: Ae, amount of drug in urine; $AUC_{0\text{-inf}}$, area under the plasma concentration–time curve from 0 h to infinity; CI, confidence interval; CL_R, renal clearance; C_{max}, maximal plasma concentration; CL/F, oral clearance; IQR, interquartile ranges; GMR, geometric mean ratio; P-gp, P-glycoprotein; T_{max}, time to maximum plasma concentration; $T_{½}$, elimination half-life.
[a]For 1 healthy adult, we obtained urine from 0–10 h and 10–32 h.
[b]The analysis of pharmacokinetic parameters on day 28 is based on 9 healthy volunteers since 1 had concentration values below the limit of quantification.

28 days of dicloxacillin treatment (Figure S3). $AUC_{0\text{-inf}}$ and C_{max} of dabigatran etexilate were reduced after 10 days of treatment to a GMR of 0.32 (95% CI: 0.13–0.79) and GMR of 0.43 (95% CI: 0.23–0.82), respectively. After 28 days of dicloxacillin treatment, $AUC_{0\text{-inf}}$ and C_{max} for dabigatran etexilate were reduced to a GMR of 0.62 (95% CI: 0.17–2.3) and GMR of 0.78 (95% CI: 0.35–1.8), respectively (Table 1, Figure 2).

3.2 | In vitro study

In LS174T cells, dicloxacillin led to a concentration-dependent increase in mRNA expression of P-gp (*ABCB1*) after 72 h of exposure relative to the control (E_{max} of 3.5-fold, EC_{50} of 328 μM; Figure 3, Table 2). We did not find a concentration-dependent increase in mRNA of P-gp (*ABCB1*) following exposure to flucloxacillin (Figure 3, Table 2). In 3D spheroid PHH, exposure to dicloxacillin for 96 h led to a concentration-dependent increase in mRNA expression of P-gp (*ABCB1*; E_{max} of 1.6-fold, EC_{50} of 19.2 μM), and flucloxacillin increased expression of P-gp (*ABCB1*; mRNA) with E_{max} of 1.5-fold and EC_{50} of 1.9 μM (Figure 3, Table 2). Dicloxacillin increased the mRNA expression of BCRP (*ABCG2*), *CES1* and MRP2 (*ABCC2*; also protein expression) in 3D spheroid PHH. Flucloxacillin increased mRNA and protein expression of MRP2 (*ABCC2*) in 3D spheroid PHH (Figure S4, Table S1). Dicloxacillin and flucloxacillin did not increase expression of OATP1B1 (*SLCO1B1*), OCT1, sodium-taurocholate cotransporting polypeptide or MRP6 in 3D spheroid PHH (Figure S5).

3.3 | Pharmacoepidemiologic study

We included 39 231 individuals with concomitant use of DOACs and dicloxacillin (Table S2) from Danish registries, matched 1:1 to users of DOACs and phenoxymethylpenicillin, and 22 488 DOAC–flucloxacillin users from CPRD matched 1:1 to DOAC–amoxicillin users (Table S3). The propensity score matching resulted in well-matched cohorts. In Danish data, a total of 113 strokes/systemic embolisms were reported among DOAC–dicloxacillin users, compared to 121 among DOAC–phenoxymethylpenicillin users (Figure 4). The HR for DOAC–dicloxacillin *vs.* DOAC–phenoxymethylpenicillin was 0.93 (95% CI: 0.72–1.2; Table S4). When matched 1:2 to nonuse of antibiotics, we found an HR of 1.2 (95% CI: 0.94–1.5; Table S4). In CPRD, 24 strokes/systemic embolisms were reported for DOAC–flucloxacillin users compared to 27 among DOAC–amoxicillin users (Figure 4). The HR for flucloxacillin *vs.* amoxicillin was 0.89 (95% CI: 0.51–1.5; Table S4). Compared to nonusers of antibiotics, HR was 0.81 (95% CI: 0.50–1.3; Table S4).

When stratifying our data to the individual DOACs among dicloxacillin and flucloxacillin users, we did not find an increased risk of stroke/systemic embolism (Table S4). Further, we found no exacerbated risk after extending the observation period to 5–30 days (Table S4) or when stratifying patients by sex or age (Table S5, Table S6). The risk of bleeding was not changed in the dicloxacillin or flucloxacillin cohort (Table S5, Table S6). In the Danish case crossover study for dicloxacillin, the odds ratios (ORs) were slightly elevated for stroke/systemic embolism both among DOAC–dicloxacillin users (OR: 1.5, 95% CI: 1.3–1.8) and DOAC–phenoxymethylpenicillin users

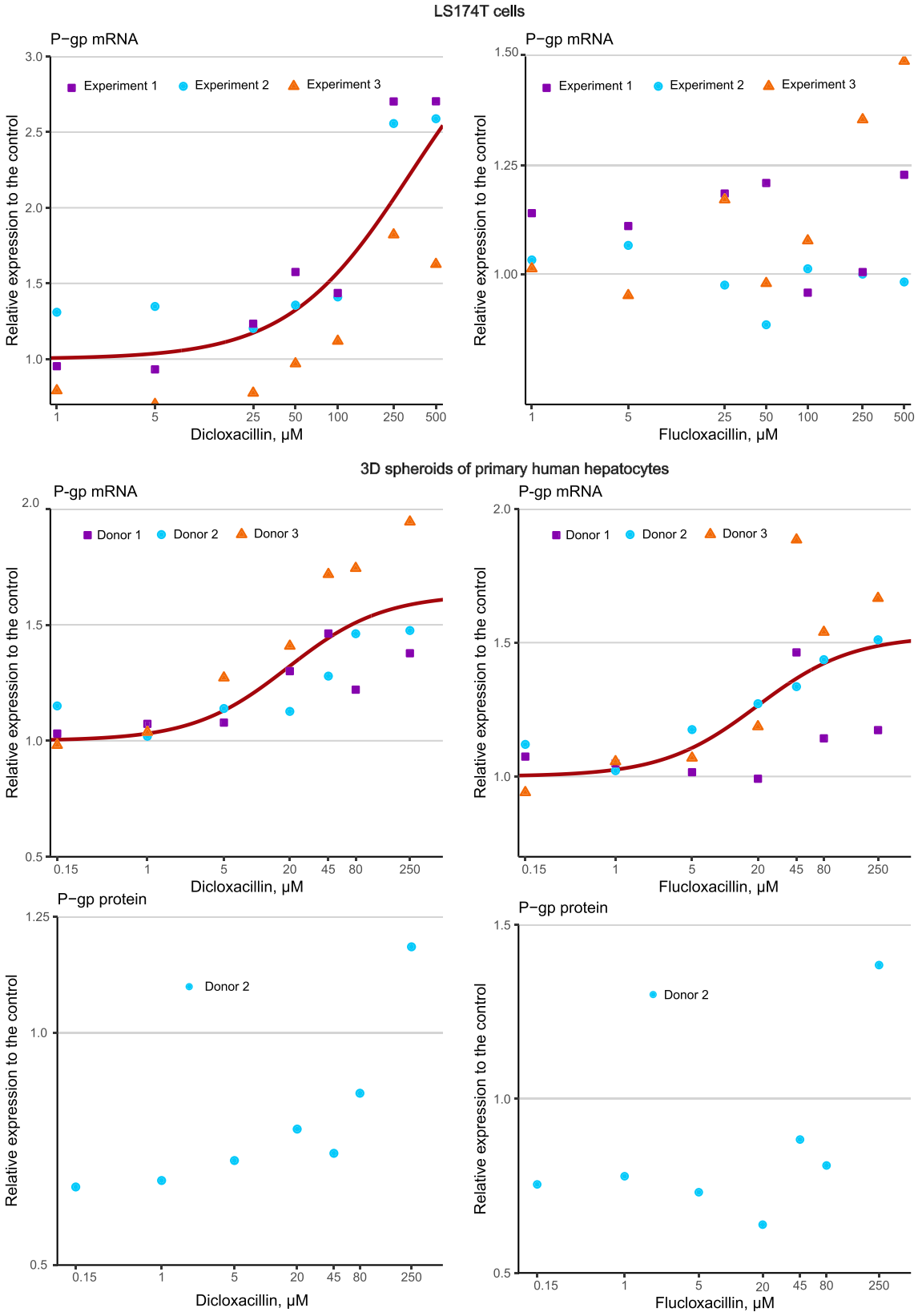

FIGURE 3 Dicloxacillin increases expression of P-gp (*ABCB1*) in LS174T cells. In 3D spheroid of primary human hepatocytes (PHH), both dicloxacillin and flucloxacillin increased the expression of P-gp (*ABCB1*). In LS174T cells, $n = 3$ experiments are represented as mean values of triplicate (experiment 1) and duplicate (experiment 2 and 3) pools with duplicate technical replications. In 3D spheroid PHH, $n = 3$ donors for mRNA and 1 donor for protein are each represented as mean values of technical replicates. P-gp, P-glycoprotein.

TABLE 2 In vitro, LS174T cells show that 72 h of exposure to dicloxacillin increases expression of P-glycoprotein (P-gp; ABCB1). Expression of P-gp (ABCB1) after 72 h of flucloxacillin exposure did not increase. 3D spheroid of primary human hepatocytes (PHH) shows that 96 h of exposure to dicloxacillin or flucloxacillin increases the expression of P-gp. In LS174T cells, $n = 3$ experiments are of triplicate (experiment 1) and duplicate (experiment 2 and 3) pools with duplicate technical replications. In 3D spheroid PHH, $n = 3$ donors for mRNA and 1 donor for protein are each represented as mean values of technical replicates.

		LS174T cells			
		mRNA		Protein	
		E_{max} (95% CI)	EC_{50} (95% CI)	E_{max} (95% CI)	EC_{50} (95% CI)
Dicloxacillin	P-gp (*ABCB1*)	3.5 (2.7–5.7)	328 μM (150–976)	NA	NA
Flucloxacillin	P-gp (*ABCB1*)	-	-	NA	NA
		3D spheroids of primary human hepatocytes			
		mRNA		Protein	
		E_{max} (95% CI)	EC_{50} (95% CI)	E_{max} (95% CI)	EC_{50} (95% CI)
Dicloxacillin	P-gp (*ABCB1*)	1.6 (1.5–1.8)	19.2 μM (7.2–45.1)	-	-
Flucloxacillin	P-gp (*ABCB1*)	1.5 (1.4–1.7)	19.7 μM (5.7–55.2)	-	-

Abbreviations: CI, confidence interval; EC_{50}, the half-maximal effect concentration; E_{max}, maximum effect; NA, not measured; P-gp, P-glycoprotein, - indicates that experiment was performed but it was not possible to calculate EC_{50} or E_{max}.

(OR: 1.4, 95% CI: 1.2–1.6; Table S7). An increased risk was also observed in the case crossover study for flucloxacillin in CPRD, with an elevated OR among DOAC–flucloxacillin users (OR: 1.2, 95% CI: 1.0–1.6) and DOAC–amoxicillin users (OR: 1.3, 95% CI: 1.1–1.5; Table S7).

4 | DISCUSSION

We found that dicloxacillin reduces oral absorption of dabigatran in healthy adults and increases the expression of intestinal P-gp but not hepatic P-gp at clinically relevant doses in vitro. Flucloxacillin did not increase the expression of intestinal or hepatic P-gp in vitro. Neither short-term use of dicloxacillin nor flucloxacillin increased the risk of stroke/systemic embolism in patients treated with DOACs. Collectively, this indicates that dicloxacillin is an inducer of intestinal P-gp, while flucloxacillin is not considered as such.

We have previously shown that combining dicloxacillin or flucloxacillin with warfarin reduced the therapeutic efficacy and increased the risk of stroke/systemic embolism.[5–7] While we provide evidence of a plausible mechanism for a similar interaction between dicloxacillin/flucloxacillin and DOACs, this pharmacokinetic drug–drug interaction did not translate into clinically manifested increased risks. This indicates that DOACs are a safer option compared to warfarin when coadministered with dicloxacillin or flucloxacillin. This might be due to the wider therapeutic range of DOACs,[22] which means that even if their plasma concentrations are reduced by concomitant treatment with dicloxacillin/flucloxacillin, their effectiveness is not substantially compromised.

We previously found that dicloxacillin also induces CYP3A4, which has a large substrate overlap with P-gp.[1,9] This dual induction of CYP3A4 and intestinal P-gp by dicloxacillin can potentially affect a wide range of drugs, leading to an exacerbated interaction and lower clinical efficacy of such drugs (e.g., tacrolimus and cyclosporin).[23] Flucloxacillin is known to interact with tacrolimus (CYP3A4 and P-gp substrate), posaconazole (**uridine glucuronosyltransferase** [**UGT1A4**] and P-gp substrate), voriconazole (CYP2C19 and minorly CYP2C9 and CYP3A4 substrate), and repaglinide (**CYP2C8** and CYP3A4 substrate).[24–28] As dicloxacillin is a stronger CYP3A4 inducer and probably a stronger P-gp inducer, it is expected to cause more pronounced interaction with these drugs.[8,9] The plasma concentration of intestinal P-gp substrates will decrease upon induction of P-gp. To overcome these clinical consequences, the dose of intestinal P-gp substrates with narrow therapeutic indices may be considered temporarily increased when administered with dicloxacillin. The dose increase should be maintained until induction of P-gp gradually wears off upon discontinuation of dicloxacillin. This is to avoid rebound effects after patients discontinue treatment with dicloxacillin. Additional research is required to fully elucidate the clinical impact of these potential drug–drug interactions with dicloxacillin.

For both dicloxacillin and flucloxacillin, the expression of P-gp increased in liver cells in vitro; however, this occurred at concentrations that exceeded plasma levels following oral ingestion of the drugs in humans. Consequently, neither drug is considered to induce hepatic P-gp. This conclusion aligns with a study on the stronger CYP3A4 and P-gp inducer rifampicin, which only induced intestinal P-gp.[29]

Discrepancies in the incidence rate of stroke/systemic embolism were evident when comparing the Danish and British cohorts, yet consistent with previous studies on Danish and British data.[30,31] Notably, the incidence rate for stroke in Denmark surpassed that in the UK. Several factors might contribute to these observed variations. Firstly, divergent data sources could contribute to different incidence rates. The British database contains data from general practitioners and differs from the Danish database, which relies on hospitalization

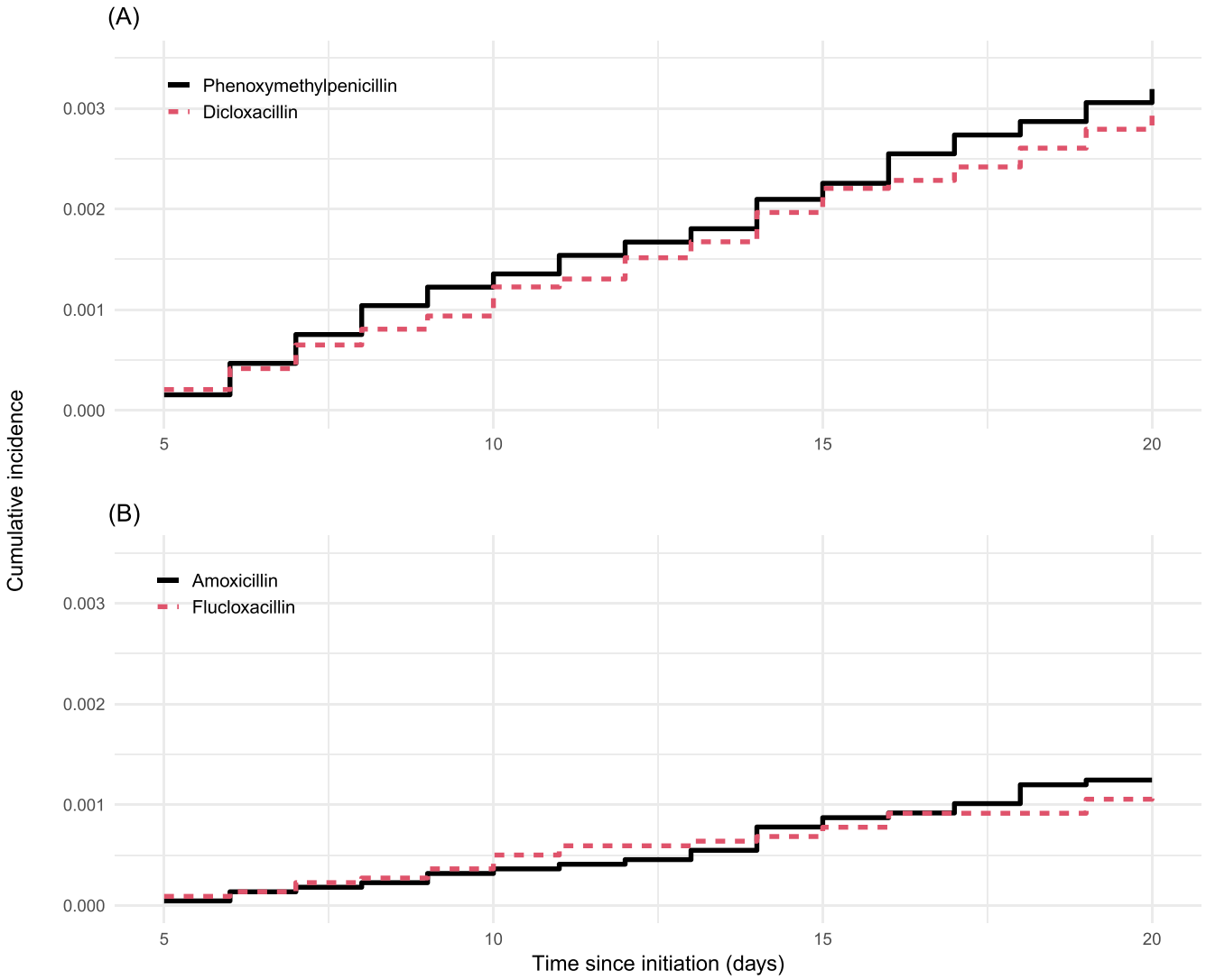

FIGURE 4 Kaplan–Meier plot from 5–20 days risk of stroke/systemic embolism in patients taking direct oral anticoagulants cotreated with (A) dicloxacillin or phenoxymethylpenicillin or (B) flucloxacillin or amoxicillin.

records. This variation in data origin might cause lower ascertainment of outcome measures from general practitioner offices. Secondly, an examination of Charlson comorbidity index and $CHAD_2DS_2$-VASc-score across the cohorts revealed higher scores in Denmark, indicating a higher extent of comorbidities and, therefore, an inherently higher risk of stroke among DOAC users in Denmark than in the UK. Finally, the Danish data on drug exposure relies on prescriptions dispensed/filled, whereas the UK data are prescription-only data.

Our findings in the pharmacoepidemiologic study are limited to patients treated with dicloxacillin/flucloxacillin for a short period. We exclude patients who redeemed a prescription of >200 capsules of dicloxacillin/flucloxacillin, as they are likely to be treated for endocarditis or osteomyelitis, and our control group did not account for this indication. We deemed it necessary to exclude these patients since phenoxymethylpenicillin is not used against endocarditis and osteomyelitis, and, thus, we would have no appropriate control group. As such, it remains uncertain whether a combination of DOAC with long-term use of dicloxacillin/flucloxacillin is safe. However, from a pharmacokinetic point of view, we do not expect that long-term treatment with antibiotics would increase the risk of stroke/systemic embolism among DOAC users. Firstly, 28 days of dicloxacillin treatment did not lead to exacerbated induction of intestinal P-gp in this study. Furthermore, DOACs have short elimination half-life and quickly reach onset and offset of action. This means that a single day without intake of DOAC can leave the patient at subtherapeutic levels.[12] This contrasts with warfarin, where long-term treatment with dicloxacillin leads to reduced efficacy.[6,12,32] This exacerbated interaction might be explained by warfarin's slower onset of action and longer elimination half-life.[33]

The main strength of this study is the translational approach used to investigate the induction of P-gp and the consequences in a real-world setting using Danish and British electronic healthcare databases. We investigated both short- and long-term effects of dicloxacillin on the concentration of dabigatran and dabigatran etexilate in the clinical trial. We measured mRNA expression and protein abundance for drug transporters in cell models of both intestine and

liver. We used electronic healthcare databases with high coverage and validity in our cohorts.[34–36] There are, however, also several limitations to consider. In the clinical trial, we cannot ensure that the reduction in dabigatran concentration is only caused by intestinal P-gp induction. To fully confirm the induction of intestinal P-gp, intestinal biopsies are necessary. We do not find it ethical to perform such a surgical intervention on healthy adults and thus decided against this. Dabigatran etexilate concentrations were very low, and several patients had concentrations below the limit of quantification in plasma shortly after intake of dabigatran etexilate, which limits the interpretation. The main limitation of the extensive in vitro work is that we did not perform activity assays, which are more appropriate indicators of function than gene regulation. Another limitation is that LS174T cells is a colorectal cancer cell line[37]; however, looking at previous studies, this cell line is suitable for investigating induction of P-gp in the intestine.[37] In the pharmacoepidemiologic study, we only have data for short-term treatment with dicloxacillin and cannot definitively exclude an increased risk of stroke/systemic embolism after long-term treatment with dicloxacillin.

In conclusion, intestinal P-gp expression increased at physiologically relevant concentrations of dicloxacillin. Induction of intestinal P-gp by dicloxacillin may only be clinically relevant for intestinal P-gp substrates with a narrow therapeutic range, and a temporary dose adjustment of these substrates may be necessary. Therefore, further investigation is needed on the treatment of dicloxacillin with specific intestinal P-gp substrates with narrow therapeutic ranges. However, DOAC users are not at increased risk of stroke/systemic embolism when they are cotreated with dicloxacillin/flucloxacillin.

AUTHOR CONTRIBUTIONS

Ditte B. Iversen, Ann-Cathrine Dalgård Dunvald, Shahab Abtahi, Olaf Klungel, Kim Brøsen, Per Damkier, Anton Pottegård and Tore B. Stage designed the research. Ditte B. Iversen, Ann-Cathrine Dalgård Dunvald, Glenn Brøde Jeppesen and Erkka Järvinen performed the research. Flemming Nielsen, Helen S. Hammer and Oliver Pötz contributed with new reagents/analytical tools. Ditte B. Iversen, Martin Thomsen Ernst, Shahab Abtahi, Patrick Souverein, Glenn Brøde Jeppesen and Erkka Järvinen analysed the data. Ditte B. Iversen, Anton Pottegård and Tore B. Stage wrote the manuscript. All authors reviewed the paper and approved the final version.

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CONFLICT OF INTEREST STATEMENT

Ann-Cathrine Dalgård Dunvald has given paid lectures for Astellas Pharma and Tore B. Stage has given paid lectures for Pfizer and Eisai and done consulting for Pfizer, and collaborated with Novo Nordisk A/S, all unrelated to the work reported in the present paper. Oliver Pötz is a shareholder of SIGNATOPE GmbH. SIGNATOPE offers assay development and service using mass spectrometry-based immunoassay technology. The rest of the authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data underlying this article cannot be shared publicly due to the privacy of individuals who participated in the study. The in vitro data will be shared on reasonable request to the corresponding author.

PATIENT CONSENT STATEMENT

Healthy adults consented to participate in the clinical trial before being included.

CLINICAL TRIAL REGISTRATION

The trial was registered at http://www.clinicaltrials.gov (identifier NCT05073627).

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