Circadian Variation in the Response to Vaccination: A Systematic Review and Evidence Appraisal

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Abstract

Molecular timing mechanisms known as circadian clocks drive endogenous 24h rhythmicity in most physiological functions, including innate and adaptive immunity. Consequently, the response to immune challenge such as vaccination might depend on the time of day of exposure. This study assessed whether the time-of-day of vaccination (TODV) is associated with the subsequent immune and clinical response by conducting a systematic review of previous studies. The Cochrane Library, Pubmed, Google, Medline and Embase were searched for studies that reported time-of-day of vaccination and immune and clinical outcomes, yielding 3,114 studies; 23 of which met the inclusion criteria. The global SARS-CoV-2 vaccination programme facilitated investigation of TODV and almost half of the studies included reported data collected during the COVID-19 pandemic. There was considerable heterogeneity in the demography of participants and type of vaccine and most studies were biased by failure to account for immune status prior to vaccination, self-selection of vaccination time, or confounding factors such as sleep, chronotype and shiftwork. The optimum TODV was concluded to be afternoon (five studies), morning (five studies), morning and afternoon (1 study), midday (1 study) and morning or late afternoon (1 study) with the remining 10 studies reporting no effect. Further research is required to understand the relationship between TODV and subsequent immune outcome, and whether any clinical benefit outweighs the potential effect of this intervention on vaccine uptake.

1. Introduction

In 1798, Edward Jenner reported that infection with cowpox conferred immunity to smallpox, an observation that yielded a prophylactic tool that would eliminate the disease from the world by 1980. (Edward Jenner, 1798) Today, vaccination is a key component of primary health care and a human right that prevents around 4-5 million deaths per year as reported by the WHO (2023). However, at an individual level, the effectiveness of vaccination can be compromised by poor immunological responses in those most vulnerable to infection, including older adults, those who are immunocompromised and people with obesity (Zimmermann & Curtis, 2019). Interventions that enhance vaccine effectiveness could help to improve clinical outcomes and to optimise the control and global elimination of infectious disease.

Circadian rhythms are daily oscillations in physiology that are driven by feedback loops in the transcription and translation of a panel of "clock" genes and other biochemical timing mechanisms that are present in virtually every human cell, including immune cells (Takahashi, 2017). The concept of circadian rhythmicity in immunity implies that there are times of day that immune defence and resilience to infection are heightened and survival is optimised. In support of this, daily windows of increased susceptibility to viral infection, and to the lethal effects of sterile inflammatory challenge have been demonstrated in animal models (Edgar et al., 2016; Halberg et al., 1960; Sengupta et al., 2019).

Similarly, the immune response to vaccination has been shown in animal studies to be dependent on the time of day; mice vaccinated towards the end of their resting phase (day-time) showed increased T-cell activation and proliferation (Fortier et al., 2011; Ince et al., 2023; Nobis et al., 2019), migration of dendritic cells into the lymph nodes (Holtkamp et al., 2021), germinal centre B-cells and circulating antibodies (Ince et al., 2023) compared to those vaccinated in their active phase. However, there is conflicting evidence for the optimum TODV in mice, with some studies reporting increased antigen-specific lymphocyte proliferation (Silver et al., 2012) elevation of antigen-specific antibodies, germinal centre B-cells, and follicular helper T (Tfh) cells (Suzuki et al., 2016) after vaccination in the active (night-time) phase. Most of these animal studies demonstrated persistence of the effects of TODV in constant conditions and abrogation or attenuation in clock-deficient animals, supporting direct regulation of the immune response to vaccination by the circadian clock.

There is accumulating evidence for comparable circadian variation in human immune function; circadian oscillation in clock gene expression have been demonstrated in human peripheral blood mononuclear cells (Boivin et al., 2003), and in CD4+ T-cells (Bollinger et al., 2011) and functional immune rhythms were suggested by diurnal patterns in IL-2, IL-4, and IFN-γ production in *ex vivo* stimulated human CD4+ T-cells (Bollinger et al., 2011). There is considerable evidence for circadian regulation of the innate immune system, including circadian oscillation of clock gene expression in phagocytic cells (Nguyen et al., 2013; Timmons et al., 2020), and variation in recruitment of neutrophils to sites of inflammation (Gibbs et al., 2014).

Studies in UK Biobank reported population-level diurnal variation in white blood cells and inflammatory markers that were independent of demographic and lifestyle confounding factors (Wyse et al., 2021). Despite the convincing evidence of the importance of TODV in mouse models, there is much discrepancy between studies of the timing of vaccination in human medicine. In contrast to animal models, the assessment of the effect of TODV in humans is confounded by many lifestyle factors that show daily variation (e.g., work, stress, mealtimes, antigen exposure) that could mask an effect of endogenous circadian rhythms in immune function on response to vaccination. Furthermore, the time-of-day preference of an individual (chronotype) is associated with genetics (Jones et al., 2019), health and age (Knutson and von Schantz, 2018) and could link the TODV to vaccination outcome independent of any underlying circadian rhythm in immune function. Assessment of an effect of TODV in humans must account for multiple confounding factors that affect human immune function such as age (Wu et al., 2022), sex (Zimmermann & Curtis, 2019), sleep (Lange et al., 2011), shift work (Ruiz et al., 2020), vaccination history (Tsang et al., 2014), and co-morbidity (Zimmermann & Curtis, 2019).

Time-dependent responses to vaccination might be caused by endogenous rhythms that serve to optimise immune function at specific times of day. Vaccination is an elective immune challenge that could theoretically be aligned with an optimal circadian phase to improve effectiveness, but this would also present a logistic obstacle to mass-vaccination and could undermine public confidence in vaccination at times of day proposed to be less favourable. Here we report a systematic review of studies that investigated human immune response to

vaccination at different times of day and assess the evidence to support diurnal variation in the effectiveness of vaccination.

2. Methods

2.1 Literature searches

A protocol for this review was registered in the International Prospective Register of Systematic Reviews (PROSPERO, CRD42023401086) and this review is reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Matryba et al., 2022). We searched the following three databases; Pubmed, Embase, Cochrane Library and Medline with no restriction on the time of publication. The search was limited to the English language and included preprint publications and theses. The search terms and MESH headings for all databases are available in the supplementary material. The reference lists of relevant reviews and of all included studies were hand-searched for additional studies. The search was designed with the aid of the following tools the Systematic Review Accelerator (https://pubmed.ncbi.nlm.nih.gov/32004673/) and the Deduplicator (https://pubmed.ncbi.nlm.nih.gov/32004673/).

2.2 Study selection

Two reviewers (CW and LR) screened the titles and abstracts of the papers retrieved by the search, and a third reviewer was consulted if the two assessments disagreed. There were no restrictions on age or time of day of vaccination, nor the type of vaccination. Studies were included if they reported any immune or clinical outcome following vaccination at a defined time of day. Categorical definition, such as morning or evening were included. Animal studies were excluded. Review papers, case studies and conference abstracts with no primary data were excluded as were editorials and opinion pieces. Clinical trials, observational, cohort and retrospective study designs were included regardless of randomisation of vaccination time. The comparison was immune and clinical response to morning vaccination against vaccination at any other time of day, and the outcome defined as change in serology, immune cell numbers, phenotype or function, infection, or local or systemic adverse effects. Studies were selected if these outcomes were assessed at least once after the first or any

subsequent dose of vaccine. Figure 1 summarises the screening and the studies eliminated at full text screening and reasons for exclusion are shown in Table S1.

2.3 Outcome measurement and data extraction

Outcome variables were (i) antibody titre post-vaccination, (ii) seroconversion, (iii) white blood cell phenotype and function, (iv) self reported adverse effects, (v) infection with the pathogen vaccinated against or (vi) hospitalisation with disease vaccinated against. Data was extracted by two reviewers (EN and CW), and included information on the year of publication, study design, period and location, study population, type of vaccine(s) and intervention(s), outcome measures, results and conclusions were extracted from the included studies (Table 1). Risk of bias was assessed with the ROBINS-I tool for non-randomised studies as described (Sterne et al., 2016) and randomised studies were assessed with the ROB2 tool for randomised clinical studies (Sterne et al., 2019).

2.4 Data Analysis

The studies included in this review differed in terms of the disease vaccinated against, the type of vaccine (live, inactivated, mRNA), and the viral strains incorporated. Within those studies that did investigate the same vaccine there was no consistency between the dose studied or the interval between doses, both factors expected to affect the response to vaccination much more strongly than the TODV. Due to this heterogeneity, it was not considered appropriate to attempt a meta-analysis and a narrative synthesis approach was employed. The size of the effect of TODV relative to other factors affecting vaccination outcome was presented graphically where these data were available.

3. Results

3.1 Yield of literature search

The initial search yielded 3,114 studies. Title and abstract searches resulted in exclusion of 2,501 records, and 582 duplicates were removed, leaving 33 studies for full text review. A further 11 studies were excluded at this stage and one study was retrieved through hand-searching (see Table S1 for details). A total of 23 studies met all criteria and were selected for inclusion in the systematic review (Figure 1). Details of these studies are given in Table 1.

The 23 eligible studies were published between 1967-2023 and reported results of studies carried out in 12 countries including US (n = 4), UK (n = 5), China (n = 3), Germany (n = 3), Australia (n = 1) and other European countries (n = 5). There were 388,714 participants (range 26 – 308,481; mean ± sd 16,196 ± 36,208) in 22 studies, with one study (Hazan et al., 2023) considered an outlier in terms of numbers of participants (n = 1,515,754).

The study settings were mostly healthcare or research-based; hospital/clinic (n = 8), public health service (n = 6) and university/research institute (n = 9). There were five randomised controlled trials, eight retrospective and eight prospective observational studies, and two non-randomised trials. The majority of the studies investigated the effects of TODV of SARS-CoV-2 (n = 11) or influenza (n = 7) vaccines, and the remainder investigated *Bacillus Calmette–Guérin* (BCG) (n = 1), hepatitis (n = 3), pneumococcus (n = 1), hexavalent (n = 1) and encephalitis (n = 1) vaccination (Table 1).

3.3 Participant demography

Most of the eligible studies recruited participants from the community (45%), 23% recruited healthcare workers and the remaining studies recruited students (18%), and employees (9%). The age range of participants was 12 – 74 years, with two studies including children and six studies including people aged over 60 only (Table 1). The majority of the studies had a higher proportion of female participants and six studies had more than 70% female participants (Erber et al., 2023; Filippatos et al., 2022; Long et al., 2016; Matryba et al., 2022; Nachtigall et al., 2022; Phillips et al., 2008) (Table 1). One study had 100% male

participants (Feigin et al., 1967) Some studies reported that women were more likely to participate in studies of TODV, more likely to report adverse reactions to vaccination (Nachtigall et al., 2022), and more likely to have a higher antibody titre post vaccination than men (Nachtigall et al., 2022; Wang et al., 2022).

There was low or poorly documented ethnic diversity in the 23 studies; six studies gave details of the ethnicity of participants and the majority of their participants were White (Abbaspour et al., 2022; Jolliffe et al., 2022; Langlois et al., 1995; Matryba et al., 2022; Phillips et al., 2008; Whittaker et al., 2022). Just one study included ethnicity as a covariable in a multivariable analysis of the association between TODV and outcome (Jolliffe et al., 2022).

Work status could be implied from studies in the workplace (university, hospital, etc) (9/23 studies), but only one study electively accounted for this factor (Jolliffe et al., 2022). Three studies of people of working age accounted for shiftwork through exclusion or adjustment (Erber et al., 2023; Matryba et al., 2022; Yamanaka et al., 2022).

There were some reports of associations between demographic factors and the TODV. In one study, younger people tended to select either early morning or late afternoon appointments (Kurupati et al., 2017). In a UK-population wide study, people vaccinated against COVID-19 in the morning tended to have fewer co-morbidities (Jolliffe et al., 2022), while in a similar study in Israel, the participants vaccinated in the morning tended to have more co-morbidities and to be older (Hazan et al., 2023). Just one study considered the effect of chronotype and reported no association with vaccination outcome (Matryba et al., 2022). One study considered circadian timing; Bohn-Goldbaum et al., (2022), reported no association between the interval between vaccination and wake time and adverse events post-vaccination. The associations between TODV and outcome was thought to be stronger in aged participants in two studies (Kurupati et al., 2017; Liu et al., 2022), although there was considerable variability among vaccine types. One study reported that the effects of TODV were stronger in women (Liu et al., 2022), and another in men (Erber et al., 2023).

3.4 Vaccination history and baseline immune status

Immune status at baseline were accounted for in most studies by measuring antibody titres before vaccination, and/or by reporting previous vaccination and infection history but six studies did not assess prior vaccination or infection status at baseline (Abbaspour et al., 2022; Bohn-Goldbaum et al., 2022; Kurupati et al., 2017; Langlois et al., 1995; Long et al., 2016; Phillips et al., 2008; Zhang et al., 2021) (Table S2). Some studies reported that there was already a significant difference in immune status (antibody titre or B-cell subsets) between morning and afternoon/evening groups before the vaccine was administered (Kurupati et al., 2017; Long et al., 2016; Zhang et al., 2021).

There was no consistency in the treatment of participants that remained seronegative after vaccination between studies, some studies performed sub-group analysis (Jolliffe et al., 2022; Matryba et al., 2022), but most studies gave no information about how data from participants that did not respond to vaccination were analysed. The dose of vaccine used varied widely between studies; immune response to the first vaccine dose were reported in ten studies, to the second in three studies, and four studies reported data on combinations of response to multiple doses of vaccine. There was no information on the dose administered in six studies (Table 1). Most study durations spanned more than 6 months (14 ± 11 months; mean ± sd), and six studies were completed over 2 years or more (Hazan et al., 2023; Kurupati et al., 2017; Langlois et al., 1995; Long et al., 2016; Phillips et al., 2008; Pollmann & Pollmann, 1988).

3.6 Definition and allocation of TODV

There was considerable variation in the definition of TODV (Figure 2); three studies reported TODV as a continuous variable, two as a binary or categorical am/pm or morning/afternoon variable and the remainder reported morning and afternoon/evening as a time interval defined by clinic times or by unjustified decisions (Table 1). Across all studies, the times of morning vaccination ranged between 6am and 1pm, afternoon between 12pm and 6pm, and evening between 4pm and 10pm (Figure 2). Some studies that assessed the effect of TODV on outcome at more than two timepoints reported that the relationship was non-linear (Filippatos et al., 2022; Wang et al., 2022), or in the case of continuous measurements, reached a peak and trough within 12 hours (Erber et al., 2023; Hazan et al., 2023; Langlois et al., 1995).

There were five studies that randomised participants to receive either morning or afternoon vaccination (Gottlob et al., 2019; Karabay et al., 2008; Lai et al., 2023; Long et al., 2016; Zhang et al., 2021), three studies allowed self-selected TODV (Phillips et al., 2008; Whittaker et al., 2022; Yamanaka et al., 2022) and in two studies TODV was allocated by an administrator (Erber et al., 2023; Zhang et al., 2021). In all other studies there was no information on how the TODV was allocated (Table S2). In two of the five randomised studies (Phillips et al., 2008; Whittaker et al., 2022) 30% of participants were allowed to switch intervention (TODV) after allocation which invalidated the randomisation procedure. In all other studies there was no information about whether switching between interventions (ie between morning or evening TODV) was permitted.

3.7 Immune outcomes

The immune outcome considered (see Table 1) was most commonly antibody titre post-vaccination; two studies considered seropositivity and seven reported the number of adverse events post vaccination. Infection was the outcome variable in three studies. White blood cell phenotypes and function were less commonly assessed, reported by four studies (Table 1).

3.8 Timing of post-vaccination follow up

The interval of time elapsed between vaccination and follow up differed between participants in most studies, as well as between studies. (Table S3). In studies that compared the response to vaccination against baseline measurements, five matched the circadian timing of the baseline and post-vaccination blood sample, the time of baseline and post-vaccination samples were mis-aligned in five studies, and in remaining cases the temporal alignment between baseline and follow-up samples was not clear (Table S3). In two studies, the timing of the post-vaccination blood sample was thought to affect the significance of the TODV effect on outcome (de Bree et al., 2020; Kurupati et al., 2017).

307 *3.9 Effect of TODV*

308 The data reported on the effect of TODV on immune and clinical outcomes are shown in
309 Table S2, and the range and times of day investigated in each study are shown in Figure 2.
310 The heterogeneity between studies in the types of vaccine, the TODV and the time interval
311 between vaccination and follow up precluded meaningful meta-analysis and individual data
312 from each study are given for comparison (Table S2). Over 40% of studies (10/23) did not
313 detect any beneficial TODV and three studies reported significant non-linear associations
314 between vaccination outcome and TODV. The optimum time-of-day of vaccination (TODV)
315 was concluded to be afternoon (five studies), morning (five studies), morning and afternoon
316 (1 study), midday (1 study) and morning or late afternoon (1 study) with the remining 10
317 studies reporting no effect.

318 Of the studies that reported an association between TODV and outcome of vaccination, three
319 presented data that could be used to estimate the size of this effect (Erber et al., 2023; Hazan
320 et al., 2023; Zhang et al., 2021). In one study, morning or afternoon vaccination were
321 associated with decreased probability of infection compared to evening vaccination, 0.95
322 (0.94 - 0.96) and 0.92 (0.91- 0.93), OR (95% CI; n = 1,515,754) for morning and afternoon,
323 respectively (Hazan et al., 2023). Zhang et al., (2021) reported that antibody titres were
324 significantly higher in healthcare workers (n = 67) after morning vaccination, with the
325 difference being 14.84 (7.37-24.15) AU/ml, median (IQR). Erber et al., (2023) reported
326 increased probability of lower antibody titres after vaccination at 12-1pm, (1.45 (1.12 – 1.87),
327 OR (95% CI; n= 803) compared to 9-10am. The remaining studies either report non-
328 significant effects, or did not present data on effect size. Data from two studies that reported
329 the effect size of TODV relative to other predictors of vaccination outcome are presented
330 graphically (Figure 3).

331

332 *3.10 Risk of bias*

333 Risk of bias in randomised controlled trials was assessed using the ROB2 tool (Sterne et al.,
334 2019) and non-randomised trials with the ROBINS-I tool (Sterne et al., 2016). The risk of
335 bias for all studies ranged from moderate to critical (Table 2-3) with most studies scoring
336 poorly in the domains of baseline confounding and measurement of outcomes. The main

issues identified with baseline confounding were failure to account for existing immune status prior to vaccination, comorbidity or the underlying circadian rhythmicity of immune function. All but two studies (Lai et al., 2023; Matryba et al., 2022) were considered to be biased by their failure to assess or account for individual chronotype. Most studies with self-selected TODV did not account for behavioural parameters that might determine the selected or allocated time of day, such as work status/role or geographic location. Bias in the measurement of outcomes was considered to be moderate to serious if the risk of allocation to an intervention (e.g., morning vaccination) was related to the immune status. For example, healthy working people might select TODV outside office hours, and be more likely to have good vaccination outcomes. Studies were considered to be biased in outcome measurements if there were sequential hypothesis testing of differences between timepoints, related immune outcomes or vaccine viral strains without correction for multiple comparisons. The classification of intervention was considered to be a source of bias where the definition of TODV was unclear, or not consistent between participants. In most cases, these sources of bias were acknowledged by the study authors in their discussion and the scores allocated reflect the complexities of studying human response to vaccination.

4. Discussion

This systematic review of 23 studies of circadian timing of vaccination revealed that while some studies reported an effect of TODV, there is insufficient overall evidence that administration of vaccines at different times of day affects immune outcomes. Generalising the findings of the included studies was challenging due to their heterogeneity and an overall effect and potential clinical benefit of vaccination at different times of day is not excluded.

The ROBINS-I tool was applied to assess the risk of bias in the non-randomised studies but the diversity of study designs and populations included makes comparison of bias between the included studies using this study challenging and subjective. Nevertheless, the tool did provide a quantitative framework that helped assess the sources of bias and how they were addressed in each study.

The majority of studies exhibited bias ranging from moderate to critical and there was considerable heterogeneity between studies in terms of vaccine type, dose, interval between vaccination and follow-up and outcome variables. Most studies had small sample sizes and there were no large-scale randomised controlled studies. There were two large population-level studies but these were confounded by poor definition of the factors that determined allocation to an intervention (TODV) (Jolliffe et al., 2022) and by the potential effects of social restriction during the COVID-19 pandemic on the outcome variable (infection) (Hazan et al., 2023). Participant demography was sometimes related to the TODV. In one study, younger people tended to select either early morning or late afternoon appointments (Kurupati et al., 2017) possibly to accommodate work times. The studies included in this review varied extensively in their management of factors known to strongly determine response to vaccination such as type of vaccine, baseline immune status, co-morbidity, age, interval between vaccination and follow-up and interval between doses (Lange et al., 2003; Tsang et al., 2014; Zimmermann & Curtis, 2019). It follows that their conclusions about the optimum TODV also vary, with some proposing morning, afternoon, evening and midday, and the majority failing to find evidence to support any association between TODV and outcome.

In some studies, the TODV was self-selected or could be rescheduled by the participant, which favours alignment of TODV with individual circadian rhythms, so that people with

morning chronotypes might present for vaccination earlier in the day. It is well-established that people with a daily preference for activities later in the day are likely to have more co-morbidities (Knutson & von Schantz, 2018) and harmful lifestyle behaviours, (smoking, screen use, poor diet, low physical activity), (Patterson et al., 2016) all factors that might affect vaccination outcome and confound detection of any effect of circadian rhythms in immune function (Dobaño et al., 2022; Karachaliou et al., 2022; Moncunill et al., 2022). In addition to such confounding by chronotype, self-selection allows the TODV to be inadvertently associated with vaccination outcome by demographic factors. Working status is one such factor since people in full-time employment are more likely to be younger, healthier and might select appointments at lunchtime vaccination or times outside working hours (9-5pm), but only one study electively accounted for this factor (Jolliffe et al., 2022).

Shiftwork adds a further level of complexity to studies of TODV in workers, by affecting both the outcome (response to vaccination) and the likelihood of morning vaccination. Shift workers are likely to have short sleep durations (Kecklund & Axelsson, 2016), to have more co-morbidities (Kecklund & Axelsson, 2016) and to smoke (Patterson et al., 2016) compared to day-workers, all factors that affect vaccination outcome. The work patterns and disrupted circadian rhythms of shift workers might determine their TODV where self selection or rescheduling of vaccination time was permitted. Regardless of any effect of shiftwork on the TODV (intervention) or response to vaccination (outcome), the disrupted circadian rhythms that these work patterns induce would affect the position of the optimal window for vaccination within a day should one exist. Some studies of people of working age included in this review accounted for these possibilities by excluding or adjusting for shiftwork(Erber et al., 2023; Matryba et al., 2022; Yamanaka et al., 2022) but most did not consider shiftwork at all. There is evidence that sleep deprivation in the days before and after vaccination can affect the immune response (Lange et al., 2003, 2011; Spiegel et al., 2023) and it is possible that increasing homeostatic sleep pressure through the day, and variation in sleep deprivation between participants could confound effects of TOV in studies that did not control for this factor.

Most investigations of the association between TODV and vaccination outcome are derived from studies of healthcare workers, students and university staff. The demography of these cohorts presents factors that affect TODV such as age, work schedule, access to vaccination and disrupted circadian rhythms from shiftwork or student lifestyles. Studying the TODV in

frontline healthcare workers is further affected by their increased risks of exposure to infectious disease that would affect their baseline immunity and vulnerability to breakthrough infection, as well as boost antibody levels if natural challenge occurred post-vaccination. The TODV of health care workers could be linked to their role in the health care setting if selected to accommodate shift patterns, or if blocks of vaccination appointment times were allocated to those most at risk of exposure. Most of the studies included in this review involved health care workers and/or medical students and their conclusions should be reproduced in a population sample.

Many demographic factors could confound detection of an endogenous circadian rhythm in response to vaccination through their effects on both TODV and vaccination outcome. This was illustrated in a study of a SARS-CoV-2 prophylactic intervention (BCG vaccination) where participants in the control group were significantly more likely to develop a COVID-19 infection after being administered a placebo (saline injection) in the morning compared to the afternoon (Föhse et al., 2023). The factors that influence individual allocation of TODV are multi-factorial, often related to vaccination outcome and are probably only controlled through randomised population-level studies.

Most of the studies included in this review spanned several months or even years (Hazan et al., 2023; Kurupati et al., 2017; Long et al., 2016; Pollmann & Pollmann, 1988), so that the season of vaccination and the interval between vaccination and follow up differed between participants and studies. This variation introduces bias due to endogenous seasonal variation in immune function, variation in the prevalence of circulating viral strains and different viral strains included in seasonal vaccines. One study reported that the season had a significant effect on the antibody response to vaccination, while the TODV was not significant (Jolliffe et al., 2022). There are well-established relationships between season and viral infection, and similar associations with vaccination are worthy of investigation.

Prior infection, exposure and vaccination history strongly affect response to vaccination (Moncunill et al., 2022; Wu et al., 2022; Zimmermann & Curtis, 2019) but not all studies accounted for these factors by assessing antigen-specific immune status at baseline. Circadian regulation of memory and adaptive immune responses to vaccination could be different, and antigen-specific immune status at baseline should be consistent between participants in studies of the TODV. In addition to antigen-specific immunity, previous

vaccination against unrelated pathogens could affect vaccine response through "trained immunity", where vaccination induces heterologous protection beyond the target disease (Benn et al., 2013). The interval between vaccination and follow up sampling could further confound detection of an effect of TODV when antibody titre is taken to represent the response to vaccination; this interval differed between participants as well as between studies included in this review.

A common source of bias occurred when baseline and follow-up samples were not collected at the same time of day, making putative changes related to TODV vulnerable to the effects of circadian rhythmicity in the outcome variable. Stable secretion of antibodies over 24h was assumed by most of the studies included in this review which seems at odds with the overall hypothesis that endogenous circadian regulation of leucocyte function could affect response to vaccination. Rhythmicity of outcome variables at baseline and follow-up could both affect detection of an effect of TODV but no study adequately controlled or adjusted for this complexity in clock-mediated regulation of immune function. Indeed, several studies reported a time-of-day effect on antibody levels at baseline (Kurupati et al., 2017; Long et al., 2016; Zhang et al., 2021), which suggests that either distinct immune phenotypes tend to be vaccinated at certain times of day, and/or that circadian variation in immune function is evident in the outcome variable at baseline. This circadian variation could be innate, as reported in animals (Cermakian et al., 2022), or secondary to masking by daily behavioural (e.g., work times) or physiological ultradian rhythms (e.g., cortisol).

The influence of circadian variation in antibody secretion after vaccination can only be resolved by sequential blood sampling over 24 hours at baseline, and at post-vaccination follow-up. There have been no studies to our knowledge that have taken this approach in humans, or even in mammals, but one study in fish demonstrated circadian rhythms in antibody secretion that were disrupted by vaccination (Guerra-Santos et al., 2018). While there is compelling evidence for circadian regulation of immune function in animals (Edgar et al., 2016; Fortier et al., 2011; Silver et al., 2012) and daily variation in some human immune parameters (Born et al., 1997; Wyse et al., 2021) it remains unclear whether human antibody production shows daily rhythmicity (Wyse et al., 2021). The effects of vaccination on such rhythms (if they exist) is also unknown, and all of these issues must be resolved before antibody titre can be used as a proxy measure of vaccine effectiveness in chronobiological studies. Animal studies of the effects of TODV have focused on innate

immunity, and the mechanisms through which TODV might affect long-term immune responses such as T-cell differentiation and B-lymphocyte maturation are unclear (Hemmers & Rudensky, 2015). The response to mRNA, vector and inactivated vaccines is elicited through different immune pathways that might be subject to varying degrees of circadian regulation. Consequently, the effect of TODV could be dependent on the type of vaccine, and this could account for some of the variation between the studies included in this review. Further studies are required to understand the circadian regulation of different immune mechanisms and their implication for chrono-vaccination.

It is of interest that most of the studies that assessed the effects of TODV at more than two timepoints reported associations with outcome that were non-linear, with a peak and trough within a 12-hour period, suggesting an ultradian rather than a circadian pattern (Hazan et al., 2023; Langlois et al., 1995; Wang et al., 2022). Such non-linear relationships would be missed by the majority of studies that assessed the effects of TODV at two timepoints. Previous studies of clock-regulated immune function in animal models and humans report oscillation over 24h (Curtis et al., 2014; Labrecque and Cermakian, 2015; Wang et al., 2022), and the ultradian patterns reported by studies in this review suggest that the circadian clock is not the predominant driver of the TODV effect they report. Nevertheless, endogenous timing is not excluded; there is increasing evidence supporting the existence of 12h innate oscillators that are independent of the circadian clock (Zhu & Liu, 2023). In fact, autonomous ultradian rhythms with a 12h period have been reported in the expression of mammalian genes involved in immune regulation, *Rela, Nfkb1,* and *Tnfaip3* (Pan et al., 2020). The differentiation and egress of hematopoietic stem and progenitor cells showed daily fluctuations that followed two daily peaks related to light and dark signals, (Golan et al., 2018) although an endogenous origin for these patterns was not established. Rhythms with a period of 12h arose earlier in evolution than circadian rhythms, driven by the requirements of ancient, ocean-dwelling creatures to entrain to the 12h rhythms of the tide rather than the 24h light-dark cycle that would later drive evolution of the circadian clock in terrestrial animals. Their significance in mammals is poorly understood, and their contribution to ultradian patterns in the response to TODV is purely speculative. It is more likely that ultradian patterns of response to vaccination are driven by human daily behaviour patterns that affect the allocation of TODV, whereby specific demographic groups attend for vaccination at times determined by the ultradian timing of work or social commitments, commute time, occupation, clinic opening times, or distance of residence from vaccination centres. It is also

possible that ultradian patterns in physiology generated by eating, stress or exercise times, or endogenous cortisol ultradian rhythms could affect response to vaccination.

The global vaccination programme implemented during the COVID-19 pandemic presented an opportunity to investigate the importance of TODV, but one that was critically confounded by the systems through which TODV were allocated, and the extraordinary lifestyle changes imposed during the pandemic. The world-wide restrictions on social mixing implemented to control transmission of SARS-CoV-2 (eg. social distancing, remote working, cocooning, lock-down) could affect conclusions about TODV. For example, the risk of exposure throughout the pandemic was highly variable between participants; both their TODV and their vulnerability to infection and humoral response to vaccination could have been affected by occupation, prevailing control measure, waves of infection and SARS-CoV-2 variants. In support of this, the factors usually associated with susceptibility to infection (age, co-morbidity, obesity) were protective in a population-level study of the TODV during the pandemic (Figure 3B) (Hazan et al., 2023), suggesting that the outcome measure (infection) was affected by social restriction of vulnerable people.

There was one randomised controlled study of TODV during the pandemic (that reported no effect) (Lai et al., 2023), but the factors controlling allocation of morning or afternoon vaccination in the other studies during the pandemic were self-selected or unclear. In many cases, TODV might have been driven by vulnerability to infection, so that health care workers, older people or people with co-morbidities had preferential access to appointments. Such allocation of the TODV by administrative or demographic factors (eg, vulnerability, occupation, age, area of residence) or by self-selection could seriously confound detection of circadian rhythms in the response to vaccination. An ultradian association was reported between the TODV and the likelihood of self-reporting COVID-19 infection (positive PCR test) after vaccination in a large (n ~ 1.5m) population sample during the pandemic (Hazan et al., 2023). The social restriction measures imposed during the pandemic caused variability in post-vaccination exposure to the virus between participants, and a self-reported infection outcome variable is compromised by the fact that the majority of post-vaccination infections are asymptomatic (North et al., 2022), and were likely to be missed.

4.1 Future research

There are many unanswered questions that must be addressed before consideration of the TODV in the clinical setting. Circadian regulation of vaccination outcome measures such as antibody titres must be further understood in animal models, and their relationship with disease resistance established for all vaccines. Randomised trials at population level are essential to accommodate the many demographic and environmental factors that affect both TODV and vaccination outcome in humans. The population-level studies included in this review that provided quantitative data on TODV report small effect sizes that suggest that sample sizes of several thousand participants should be recruited for future studies of TODV (Hazan et al., 2023; Jolliffe et al., 2022; Lai et al., 2023; Liu et al., 2022) although it must also be remembered that some studies detected statistically significant effects in much smaller samples of student or healthcare worker cohorts (eg Zhang et al., 2021, n = 62; Erber et al., 2023, n = 803). The advantage of large population-level studies is their power to adjust for the multiple demographic and lifestyle factors that might otherwise confound detection of an effect of TODV. Furthermore, investigation of the causal effects of daily variation in the response to vaccination will be facilitated by the availability of big datasets with rich individual-level information on health and lifestyle combined with advanced statistical and machine learning techniques. It should be considered that such population level studies would be costly as stand-alone endeavours but could easily be incorporated into clinical trials of vaccination, where the onus is on the vaccine producers to demonstrate that effectiveness does not depend on the TODV. At a mechanistic level, future research should apply free-running protocols to establish whether circadian rhythms in human immune function truly reflect endogenous clock-mediated oscillation or are secondary to other features of human behaviour and lifestyle that vary over 24 hours. Studies that include vaccination times that extend further into the night (ie after 9pm) would also be informative with respect to the role of the circadian clock in mediating time-dependent variability in the response to vaccination. Future research should also focus on the development of a simple method for assessment of human circadian phase that will allow endogenous daily variation in immunity to be linked to therapeutic benefit.

As a population as well as an individual prophylactic intervention, the benefit of time-dependent vaccination must be sufficiently great to justify its disruptive effect on the delivery of vaccination programmes. Manipulation of the TODV or “chrono-vaccination” is an intervention proposed to target those that respond poorly to vaccination such as the aged or immunocompromised (Otasowie et al., 2022) yet most information available is from studies

606 in students and healthcare workers. Further work should address this by studying the
607 implications of the TODV in these groups whose compromised immune and circadian
608 function might make their response to TODV quite different to that of healthy people.
609
610 *4.2 Strengths and Limitations*
611 The principal strength of this review is our critical appraisal of all currently available data on
612 the effect of TODV on vaccination outcome using an approach that adhered to recommended
613 quality standards for conducting systematic reviews including a comprehensive search
614 strategy and risk of bias assessment. This study also has limitations. The majority of the
615 studies included had observational, retrospective study designs and in most cases, the factors
616 controlling allocation to the intervention group (morning vaccination) were unknown. We
617 did not include studies only available as abstracts, which might have excluded emerging
618 evidence. We only included studies published in English which may have excluded relevant
619 studies. Comparison between studies was difficult due to the heterogenicity in vaccine types,
620 outcome variables and study design, and this precluded meta-analysis.
621
622 It is a limitation that cross-sectional changes in antibody titre were used to quantify response
623 to vaccination in most of the studies in this review rather than more objective methods for
624 assessment of vaccine effectiveness such as randomised, placebo-controlled, double-blind
625 trials. There is evidence to support the use of antibody titres as surrogate markers of efficacy
626 for COVID19 (Corbett et al., 2021) and influenza (Laurie et al., 2015) vaccines but these
627 tests do not reflect cellular immunity nor the influence of other factors that might affect
628 resistance to disease such as pre-existing immunity. The use of changes in antibody titre as a
629 continuous outcome variable implies a direct, quantitative relationship between disease
630 resistance and the proportional change in post-vaccination titre, which may not be justified.
631 Future studies should assess the impact of TODV on effectiveness of vaccination in
632 preventing infection or clinical disease to support findings from proxy measures of efficacy
633 such as changes in antibody titre.
634
635 *4.3 Conclusions*
636 At a population level, the efficacy of vaccination is compromised by vaccine hesitancy, a
637 refusal to access vaccines due to complacency, lack of confidence or inconvenience. Vaccine
638 hesitancy is identified by the WHO as one of the 10 threats to global health (WHO, 2022) and
639 its rise threatens to reverse progress made in eliminating infectious diseases such as measles,

polio and human papillomavirus. The Strategic Advisory Group of Experts (SAGE) on Immunization which advises the WHO on vaccination strategies reported convenience, including access to vaccination at an appropriate time and place, to be one of the three main factors that influences vaccine uptake (WHO, 2022) which underlines the importance of accurate research and communication of the clinical significance of the TODV.

Circadian timing mechanisms regulate most aspects of human physiology, and response to vaccination is not likely to be an exception given existing evidence for daily variability in other aspects of human immune function (Born et al., 1997; Wyse et al., 2021). Furthermore, studies in mouse models provide compelling evidence that TODV can affect susceptibility to vaccination (Ince et al., 2023; Nobis et al., 2019), and mechanisms fundamental to adaptive immunity weeks after the initial challenge (Fortier et al., 2011; Ince et al., 2023; Silver et al., 2012; Suzuki et al., 2016). Nevertheless, mouse models poorly represent the circadian response to vaccination in humans because they live in a pathogen-depleted environment, they lack pineal melatonin, they are nocturnal, and not subject to the same daily variation in environmental challenges as humans. In further contrast to mice, relationships between the TODV and outcome in humans could be mediated by endogenous timing mechanisms in combination with environmental factors that also vary by time-of-day (work, meal-times, commuting, stress) and randomised-controlled studies that control for these factors are required to support recommendations about TODV. Animal studies and prior evidence for circadian regulation of the human immune system provide mechanistic support for an effect of TODV on vaccination outcome that justifies consideration of TODV in future studies regardless of the uncertainty of current evidence. Chronovaccination could potentially improve response to vaccination in individuals and at population level, and the TODV should be considered in future studies of vaccine effectiveness. This review has identified multiple confounding factors that bias current evidence, as well as highlighted factors that should be considered in future studies.

Data availability

There were no original data produced during this work.

Declaration of competing interests

The authors declare no known competing interests that might have biased the work reported in this paper.

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Author contributions

All authors contributed to planning and design of the search strategy and study plan. CW and EN performed the searches and data extraction and drafted the manuscript. All authors critically reviewed the manuscript and approved the final version to be published**.**

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