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Epidemiological and clinical insights from SARS-CoV-2 RT-PCR cycle amplification values

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The SARS-CoV-2 pandemic has led to an unprecedented daily use of molecular RT-PCR tests. These tests are interpreted qualitatively for diagnosis, and the relevance of the test result intensity, i.e. the number of amplification cycles (C_t), is debated because of strong potential biases. We analyze a national database of tests performed on more than 2 million individuals between January and November 2020. Although we find C_t values to vary depending on the testing laboratory or the assay used, we detect strong significant trends with patient age, number of days after symptoms onset, or the state of the epidemic (the temporal reproduction number) at the time of the test. These results suggest that C_t values can be used to improve short-term predictions for epidemic surveillance.

Molecular testing is a key component of screening policies to control the spread of infectious diseases and the SARS-CoV-2 pandemic has led to an unprecedented testing rate using reverse transcription polymerase chain reaction (RT-PCR) assays. (1). In clinical and public health practices, RT-PCR results are qualitative for viral respiratory disease diagnostics, with reports such as ‘positive’, ‘negative’, ‘uninterpretable’, and, sometimes, ‘weakly positive’. These are based on the cycles threshold, also referred to as crossing point or crossing threshold (here denoted C_t), which corresponds to the number of PCR amplification cycles required for the fluorescent signal to rise above a positive threshold. In theory, the more abundant the genetic target in the sample, the fewer the amplification cycles required to detect it. This is why numerous studies on SARS-CoV-2 rely on C_t values to assess transmissibility (2) or study infection kinetics (3). Here, we present a cross-sectional analysis of SARS-CoV-2 RT-PCR tests performed on 2,220,212 individuals in France between January 21, 2020, and November 30, 2020 (Figure 1).

Few studies analyze C_t values at a population level. One explanation for this matter of affairs is that these are known to suffer from several, potentially strong, biases. First, sample type and sampling quality directly affect the amount of genetic material available. Second, the RT-PCR assay matters. Even the quality of the reagents used may have a significant effect on the number of amplification cycles required to achieve the same level of fluorescence for the same amount of target genetic material. Combining data from different laboratories helps to control for these sources of variation in statistical analyses. Furthermore, the larger the dataset, the more we can detect small statistical trends even after having controlled for non-informative variables.

In our analysis, we studied tests from individuals aged between 1 to 89 years old. We did not take into account tests for which key variables such as patient age, patient sex, laboratory geographical department, qualitative result, or RT-PCR assay used were unknown. Note that one test could provide more than one C_t value if containing probes targeting multiple viral genes. According to the national guidelines (4), it is recommended to focus on the most sensitive target to categorize levels of viral excretion. After removing the biologically unrealistic C_t values that were lower than 10 or larger than 45, the 95% confidence interval (95CI) of the remaining values was [16.89; 35.56] (Table S1). These correspond to 793, 479 tests from the same number of individuals.

We used a linear regression model to explore how C_t values can be explained by the following variables: patient age and sex, the number of days since the onset of the symptoms (if known), the clinical sampling site (if known), the sampling facility (if known), the RT-PCR assay used, the target gene, the test’s qualitative result, the sampling date, the temporal reproduction number of the epidemic at the sampling date (denoted R_t and estimated using the EpiEstim method (5)), and a control variable. The latter corresponds to the last digit of the patient anonymity number and is expected to be independent of the C_t value. We also included in the model an interaction term between sampling date and R_t . For this analysis, we excluded C_t values from internal controls. Univariate analyses are extremely sensitive to heterogeneity in the dataset. For instance, the age distribution from patients sampled in aged care homes is different than that from city screening facilities, and analyzing the ‘sampling facility’ factor alone could yield misleading result. This is why the analysis used here is multivariate and considers all the factors listed above simultaneously.

Overall, the linear model explained 38.8% of the variance in C_t values, and the residuals were normally distributed (Figure S2A). All the factors except the temporal reproduction number were significantly associated with C_t values using a classical 5% p-value criterion in an analysis of variance (ANOVA) with type II sums of squares. Even for the control variable, the p-value was 0.013 and patients with final digits 1 and 3 had C_t values slightly lower (-0.19 and -0.17 cycles) than patients with a 0 final digit. We

therefore set our significance thresholds to 5% of that of the control variables, *i.e.* 6.5×10^{-4} to analyze main effects (Table 1).

The intercept of the linear model indicates the average C_t value for a positive test performed with the reference assay, and all the other factors being set to their reference value. Its magnitude (19.1 cycles) is in line with clinical practice. The importance of the noise in the dataset is illustrated by the strong effect of the testing laboratory, as well as the RT-PCR assay used (Supplementary Figure S1).

Despite this high level of noise, we detected a strong effect of the qualitative result (Figure 2A), with C_t differences that were even larger than that from the laboratory effect. We also found a slightly significant difference of -1.81 cycles between the most common type of samples (nasopharyngeal) and that performed on other clinical sampling sites (mostly lower respiratory tracts, but also feces or saliva). This is likely because the latter tests were performed in patients with more severe symptoms.

The effects associated with the number of days since symptoms onset was particularly strong. For the 8.5% of the participants for whom the number of days between symptoms onset and testing dates was known, we found that the C_t gradually increases over the reported range with a maximum difference of 5.73 cycles (Figure 2B).

The effect of sex had the same order of magnitude as that of the control variable and could, therefore, be treated as non-significant. Conversely, the age factor had a strong effect with a decrease of 0.541 cycles per year (Figure 2C).

The target gene of the RT-PCR assay used also yielded a slightly significant effect. The C_t values obtained when using a probe targeting the ORF1 and S regions of the virus genome were significantly higher than when using the N gene, which was the genomic region of reference in the model (Figure 2D). This effect is consistent with the life-cycle of the virus. As stressed by (6), since coronaviruses are (+)ssRNA viruses, they use the same RNA matrix for replication and transcription, both being amplified by diagnostic assays. Furthermore, *Coronaviridae* transcripts can produce subgenomic mRNAs that lack part of the genome (7). As a consequence, and as shown in cell cultures (8), genes on the 5' end of the genome are under-represented. This is consistent with our result where assays targeting the gene on the 3' end (the N gene) tend to have lower C_t than assays targeting genes on the 5' end (the ORF1 and S genes). Note that an alternative explanation could be that some probes target more conserved areas of the SARS-CoV-2 than others (9).

Finally, we found that C_t values decreased with time (-0.797 cycle per day), but this effect was non-linear (Figure 2E). This could be due to the strong variation in testing efforts in France (Figure 1A), but also to variations in the epidemic trend. Indeed, although the R_t (inferred from hospitalization data using the EpiEstim method (5)) was not found to be significant, the interaction between the sampling date and R_t was nearly significant (Figure 2F), suggesting that a temporal analysis could yield additional insights.

The existence of a correlation between the C_t values of the tests performed in a population and R_t is consistent with population dynamics theory, which predicts that in an expanding population of infected individuals, the 'age' of the infections, *i.e.* the number of days post-infection, is skewed towards lower values (10). Since C_t values have been reported to increase over the course of an infection (3), which we confirm with this analysis (Figure 2D), it has suggested that these values could be used as an early signal to predict R_t (11).

To investigate this question, we focused on screening data collected in the general population from individuals aged from 5 to 79. We estimated the median and skewness values of the daily distribution of the C_t values. To correct for potential confounding factors, these were adjusted using a linear model (see the Supplementary Methods). We analyzed the temporal correlation between the time series with

a 7-day rolling average of this median, skew, and R_t (Figure 3). For the median C_t value, we found a significant correlation with R_t that was maximized for a 6 to 7 days delay (Figure S2). This is consistent with R_t being calculated using data from ICU-admissions, which occur with a median of 14 days after infection (12, 13), and RT-PCR screening data being obtained earlier in the infection.

To further assess the usefulness of C_t data, we used ARIMA models to predict R_t dynamics over 7 days. We compared models without any exogenous data, to models that also included exogenous time series (either median or skewness of estimated C_t values distribution, or the fraction of positive tests (I)). As expected, the prediction error made using only endogenous data (R_t) was low in periods where R_t variations were limited. Furthermore, we found that adding exogenous data improved the prediction, especially when strong shifts in R_t were occurring (Figure 3). C_t values (green and cyan dots) tended to provide a better reduction in the error of the prediction than the ratio of positive tests (purple).

This analysis of a large national database of RT-PCR tests performed in the context of a major epidemic confirms that population-level C_t values are noisy since even a linear model that features 91 degrees of freedom does not explain the majority of the variance. However, owing to the law of large numbers, we detect several effects that are in line with biological observations and with virological properties. For instance, our finding that C_t values decrease as a function of the number of days after symptoms onset is consistent with longitudinal follow-ups (3). Similarly, the difference we detect between the virus gene targeted by the RT-PCR assay used can be interpreted in the light of known differences in mRNA copy numbers between genes depending on their distance from the 3' end (8). Concerning the link between age and C_t values, although there are some mechanistic interpretations as to why virus load would increase with age (14), the evidence was mixed, with some studies reporting a decreasing trend (15) and others not (16, 17). Here, using a multivariate approach on a large dataset allows us to unravel a strong and significant decrease of C_t values with age.

A limitation our study is that although our dataset stands out by its size and its level of details, it is restricted to a single country where testing effort varies, both on a temporal and on a spatial scale (Figure 1). Performing similar analyses for other populations can therefore be particularly informative.

A promising output of this analysis is the possibility to use C_t values as an early signal to detect changes in epidemic behavior, *e.g.* R_t values. Indeed, our most robust descriptors of the epidemics originate from hospital-admission data, but these have a significant delay with the status of the epidemic since patients are hospitalized 2 weeks after infection (12, 13). The ratio of positive tests performed in the population of interest can, in theory, provide earlier insights but it suffers from strong sampling biases. We show that accounting for population-based C_t values variations can improve R_t predictions on a 7-days period. This corroborates earlier hypotheses (11) but also reveals the importance of analyzing a large-enough dataset to filter out the important amount of noise in these values. Overall, these results call for better integration of C_t values in national public health policies to monitor epidemics caused by SARS-CoV-2 but also other human viruses, especially since these data raise less ethical concerns than other sources of data such as mobility data.

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Table 1: **Main factors affecting Ct values.** We only list factors with significant effects with a 10^{-3} p-value criterion. Coefficients reflect differences in C_t . For qualitative factors, the reference value is shown.

Factor	Value	Coefficient	2.5% CI	97.5% CI
(intercept)		19.1	12.9	25.4
assay	PerkinElmer (ref.)	—	—	—
	Genefinder	12.1	10.3	13.9
laboratory	LAB_1 (ref.)	—	—	—
	LAB_122	5.42	3.79	7.05
	LAB_96	−4.8	−6.71	−2.90
result	positive (ref.)	—	—	—
	weakly positive	11.3	11.1	11.5
	negative	16.9	16.6	17.2
days post-symptoms onset	less than 4 (ref.)	—	—	—
	4 to 7	2.76	2.66	2.86
	8 to 14	4.90	4.73	5.08
	more than 14	5.73	5.43	6.03
sample	naso-pharyngeal (ref.)	—	—	—
	other	−1.81	−2.49	−1.14
age	(per year)	−0.541	−0.585	−0.497
target gene	N (ref.)	—	—	—
	ORF1	1.03	0.949	1.12
	S	1.19	0.948	1.43
date	(per day)	−0.797	−0.903	−0.691

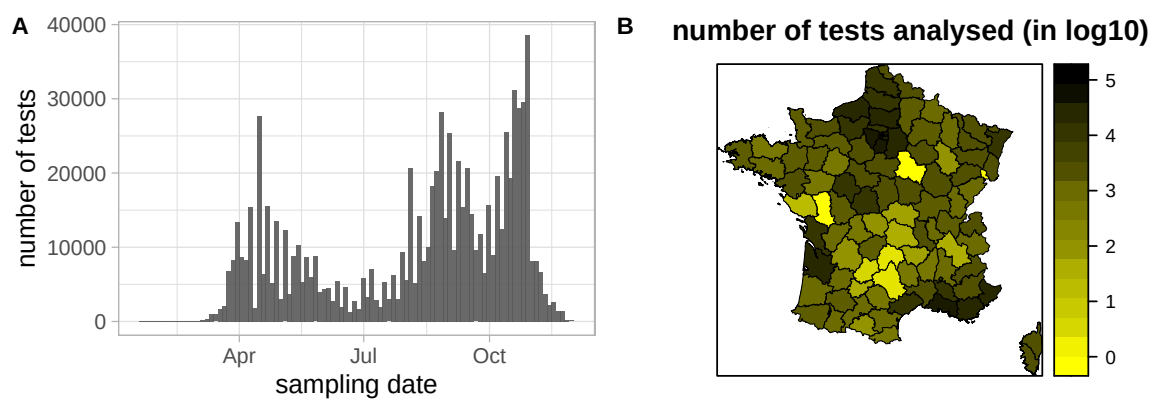


Figure 1: **Number of tests performed per day (A) and per French department (B).**

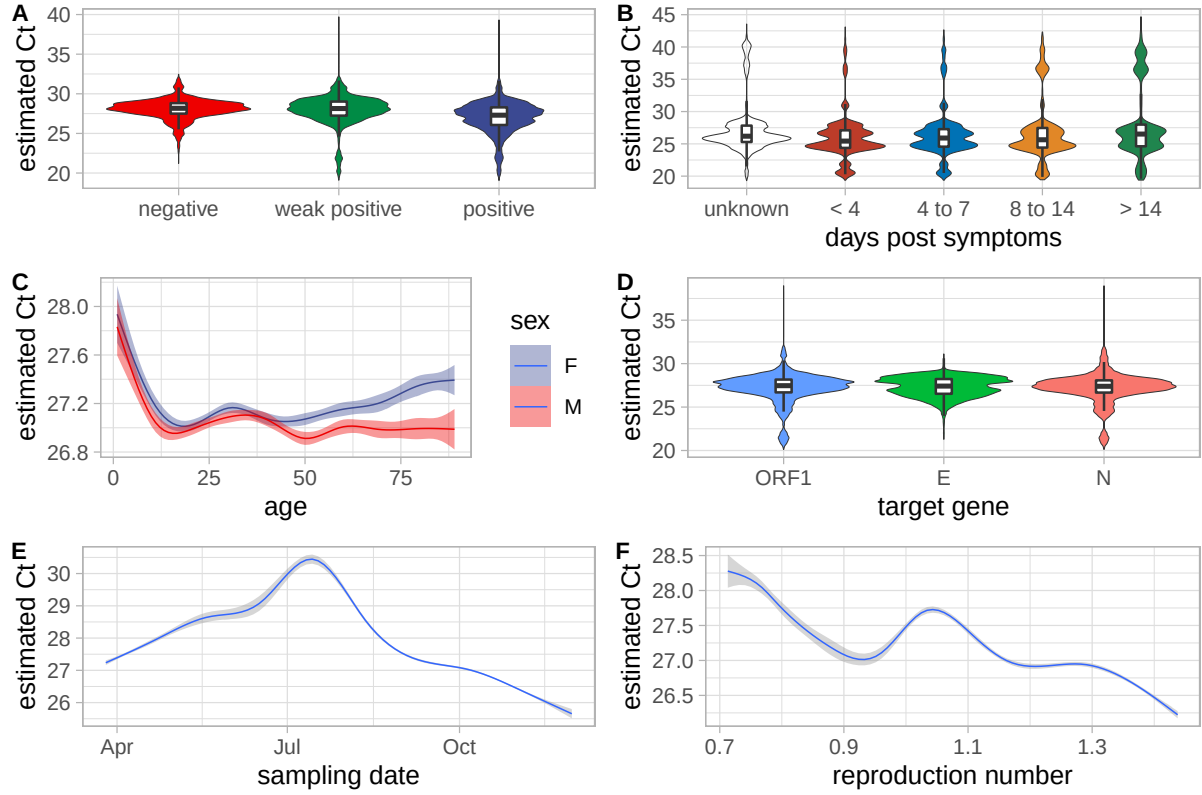


Figure 2: Correlations between key factors and observed C_t variations. A) Qualitative result of the test, B) number of days between symptoms onset and testing, C) participant age and sex, D) genomic area targeted by the test, E) sampling date, and F) temporal reproduction number (R_t) at the time of the test. For panels A, B, and D, the violin plots indicate the distributions and the box plots show the 0.025, 0.25, 0.5, 0.75, and 0.975 quantiles. Panels C, E, and F are obtained with a 'loess' smoothing model. The C_t values shown are not the raw values but that estimated using a linear model to correct for biases (see Supplementary Methods for details).

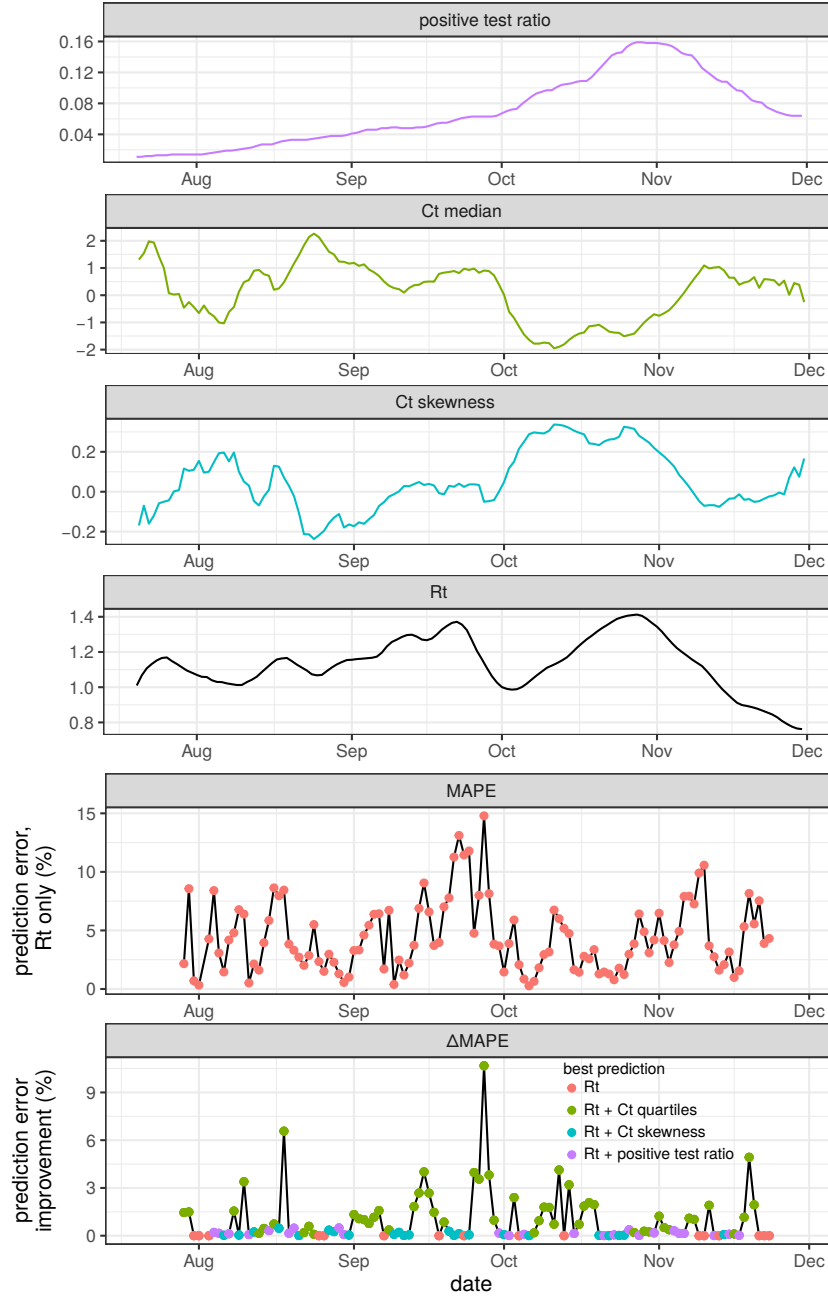


Figure 3: **Predicting temporal reproduction number (R_t) from time series.** The top four panels show the the 7-days rolling averages of the time series of the ratio of positive tests (in purple), the median (in green) and skewness (in cyan) of the daily C_t residual distribution, and R_t (in black). The bottom panels show the error made by a prediction using only R_t data (red dots) and the potential improvement made by including exogeneous data.

Supplementary materials

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S1 Authors contributions

SA, VF, and SB conceived the study, SH-B, J-MG, LN, SP, TV, AdR, CT, MS, RS, CB-B, MS, A-SLH, SB, CL, JD, SH, CH, DV, HMD, SF, BV, CH, VF, and SB contributed anonymous data, SA compiled the anonymous data sets from the collaborating partners, SA, MTS, and CS analysed the data, SA wrote the first draft of the manuscript, all authors commented and approved the manuscript.

This study was supported by the COVID-19 study group from the Société Française de Microbiologie (SFM).

S2 Ethics

This study was approved by the Internal Review Board of the Centre Hospitalier Universitaire de Montpellier. It is registered at ClinicalTrials.gov under the identifier NCT04738331.

S3 Data and scripts

The final data set analyzed along with the R scripts will be made available upon publication.

S4 SFM COVID-19 study group

The participants of the SFM COVID-19 study group are LINA Gérard (president of the SFM, CHU Lyon), VABRET Astrid (CHU de Caen), ADNET Justine (CHU de Caen), ROQUEBERT Benedicte (Cerba), DUCANCELLE Alexandra (CHU d'Angers), LE GUILLOU-GUILLEMETTE Hélène (CHU d'Angers), BOUTHRY Elise (CHU d'Angers), LUNEL-FABIANI Françoise (CHU d'Angers), PIVERT Adeline (CHU d'Angers), APAIRE-MARCHAIS Véronique (CHU d'Angers), ROGER Steven (CHU d'Angers), Chakib Alloui (Avicenne), Ségolène Brichler (Avicenne), Emmanuel Gordien (Avicenne), MIRAND Audrey (CHU de Clermont-Ferrand), ARCHIMBAUD Christine (CHU de Clermont-Ferrand), BREBION Amélie (CHU de Clermont-Ferrand), REGAGNON Christel (CHU de Clermont-Ferrand), CHABROLLES Hélène (CHU de Clermont-Ferrand), BISSEUX Maxime (CHU de Clermont-Ferrand), COMBES Patricia (CHU de Clermont-Ferrand), Hélène Jeulin (CHU de Nancy), Véronique Venard (CHU de Nancy), Evelyne Schvoerer (CHU de Nancy), Anne Lebouter (Henri Mondor APHP), Souraya Khouider (Henri Mondor APHP), Alexandre Soulier (Henri Mondor APHP), Aurelie Gourgeon (Henri Mondor APHP), Bellecave Pantxika (CHU de Bordeaux), Busson Laurent (CHU de Bordeaux), Garrigue Isabelle (CHU de Bordeaux), Lafon Marie-Edith (CHU de Bordeaux), Trimoulet Pascale (CHU de Bordeaux), Bruno Pozzetto (CHU de Saint-Etienne), Thomas Bourlet (CHU de Saint-Etienne), Sylvie Gonzalo (CHU de Saint-Etienne), Rémi Labetoulle (CHU de Saint-Etienne), Rogez Sylvie (CHU de Limoges), Alain Sophie (CHU de Limoges), Marianne Coste-Burel (CHU de Nantes), Virginie Ferré (CHU de Nantes), Berthe-Marie Imbert-Marcille (CHU de Nantes), Pierre Edouard Fournier (IHU Méditerranée infection), Petit Paul Rémi (IHU Méditerranée infection), Luciani Léa (IHU Méditerranée infection), Zandotti Christine (IHU Méditerranée infection), Charre Caroline (Cochin APHP), Mariaggi Alice-Andrée (Cochin APHP), Méritet Jean-François (Cochin APHP), Rozenberg Flore (Cochin APHP), Febreau Christine (CHU de Rennes), Comacle Pauline (CHU de Rennes), Lagathu Gisèle (CHU de Rennes), Maillard Anne (CHU de Rennes), Grolhier Claire (CHU de Rennes), Pronier Charlotte (CHU de

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S5 Supplementary Methods

S5.1 Initial data filtering

The French Society for Microbiology (SFM) send a query to collect anonymous RT-PCR test results data from 19 public and 2 private laboratories. The response files were curated manually and merged using R. Test values without any C_t values (negative tests) were removed.

This led to an initial global data set from 2,220,212 individuals. Removing non-numerical C_t values (usually a qualitative description of a negative result) decreased this number by 30% from 10,668,371 to 7,516,936 C_t values (note that most tests are usually associated with more than a single value since it can have multiple targets). We then removed all the C_t values equal to zero, which left us with 1,969,043 values.

Finally, we performed some extra filtering to remove aberrant C_t values (greater than 100), test with missing values for sampling French department, qualitative result, or RT-PCR test used. This left us with 1,299,447 values originating from 824,446 individuals. Further details are available in Table S1.

For each individual, we retained only the earliest sample therefore analyzing 1,129,437 C_t values.

S5.2 Database properties

Table S1: **Description of the dataset variables.** For real numbers, we show the median and the 95% confidence interval. For categorical variables, we either indicate the number of factors or the number of occurrences n for each factor.

Variable	Description	Details	Values
department	French administrative department where the sampling was performed	categorical	97 departments
id_lab	laboratory associated with the sampling	categorical	128 labs
control_variable	a control variable created using the last digit of the patient anonymity number	categorical	10 values
id_patient	participant anonymity number	categorical	825, 446 ids
date	sampling date	date	min = 01/21/2020 max = 30/11/2020
sampling_facility	type of facility where the sampling was performed	<i>city screening</i> <i>aged care home</i> <i>hospital</i> <i>prison</i> <i>missing</i>	$n = 1,008,307$ $n = 3,822$ $n = 72,682$ $n = 45$ $n = 44,570$
sample_type	clinical localization sampled	<i>nasopharyngeal</i> <i>other</i>	$n = 1,086,004$ $n = 35,660$
assay_PCR	the description of the RT-PCR assay used	categorical	13 assays
target_gene	SARS-CoV-2 genomic area corresponding to the C_t value	N E S $ORF1$ $N \& ORF1$ <i>internal control</i>	$n = 199,256$ $n = 55,717$ $n = 9,953$ $n = 227,192$ $n = 1,196$ $n = 636,123$
C_t	amplification cycles required to reach the signal (0 values are removed)	real	28.10 [18.30; 37.37]
R_t	temporal reproduction number (see Supplementary methods for details)	real	1.15 [0.76; 1.41]
result	qualitative result of the test	<i>positive</i> <i>negative</i> <i>weak positive</i>	$n = 524,065$ $n = 585,293$ $n = 20,079$
age	participant age (in years)	integer	48 [25; 83]
sex	patient sex	<i>female</i> <i>male</i>	$n = 637,212$ $n = 492,225$
symptoms	number of days between symptoms onset and testing	<i>less than 4</i> <i>4 to 7</i> <i>8 to 12</i> <i>more than 12</i> <i>missing</i>	$n = 107,417$ $n = 22,308$ $n = 6,070$ $n = 1,945$ $n = 991,697$

S5.3 Temporal reproduction number (R_t)

The temporal reproduction number (denoted R_t) was computed on the COVID-19 hospital admission incidence time series established by the national public health agency (Santé Publique France) and accessible at this website. Because of strong daily variations (especially on week-ends), we first transformed the time series using a 7-days rolling average. We then used the EpiEstim method (5) and the eponym package in R (18).

Earlier studies have reported that, for patients who develop severe symptoms, the median time between infection and hospital admission is in the order of 14 days (12, 13).

S5.4 Statistical analyses

All the analyses were performed in R version 3.6.3.

S5.4.1 Linear model

The linear model was performed in individuals from 1 to 90 years old. We also removed C_t values associated to internal controls because they caused the distribution of the residuals to be non-Gaussian. Quantitative factors, namely $R(t)$, date, and age were scaled and centered.

The model was formulated as follows in R:

```
modele_general = lm(Ct ~ Rt*date + age + sexe + target_gene +  
assay_PCR + id_lab + symptoms + result +  
sample_type + sampling_facility + control_variable, data)
```

The adjusted R^2 of the model was 38.8% and the distribution of the residuals Gaussian (Figure S2A).

The model was analyzed using an ANOVA assuming type-II errors because of the unbalanced nature of the data set. All the variable were found to be extremely significant (p-value $< 10^{-6}$), except for R_t (p-value of 0.68) the control variable (p-value of 0.0131), sampling facility (p-value of 0.0135), sex (p-value of 0.0021), interaction between R_t and date (p-value of 0.000842).

S5.4.2 Time series analyses

To analyze the time series of reproduction number and C_t values we restricted the data to tests performed after July 1, 2021, in a screening context, using nasopharyngeal swabs, in individuals aged from 6 to 80. These assumptions were made such that C_t values would reflect the state of the ongoing epidemic. Finally, we excluded values from internal control genes. Overall, we analysed 234,782 C_t values from 110,227 individuals.

To correct for other potential confounding factors, we first performed a linear model.

The analyses were performed on the residuals of the linear model. For each day, we computed the median and the skewness of the distribution. Skewness was computed using the formula $\frac{n}{(n-1)(n-2)} \sum_i \left(\frac{x_i - \text{mean}(x_i)}{\text{Var}(x_i)} \right)^3$, with x_i the individual values and n the total number of values. We then computed a 7-day rolling mean to buffer daily variations.

The temporal reproduction number was computed as indicated above. We also analyzed a 7-day rolling mean.

Cross-correlation function analyses were performed using the `ccf` function in R.

S5.4.3 Predictive analyses

We used ARIMA models (implemented in the R package `forecast`) to predict the hospital-admission temporal reproduction number (R_t) from past observations.

For each date, predictions were evaluated in terms of the mean absolute percentage error (MAPE) for horizons of 7 days in the future based on coefficients learned from past data starting on July 29th, 2020. More precisely, for each date we compared the temporal reproduction number data $\{D_k; k = 1, \dots, 7\}$ with the seven-day model forecast $\{F_k; k = 1, \dots, 7\}$ by

$$\text{MAPE} = \frac{1}{7} \sum_{k=1}^7 \left| \frac{D_k - F_k}{D_k} \right|$$

We considered 4 types of data:

1. past R_t data (i.e. endogenous data),
2. quartiles from C_t residuals (to remove biases),
3. skewness from C_t residuals,
4. national positive test ratio collected from <https://covid.ourworldindata.org/data/>.

The residuals of C_t were obtained from the following linear model:

```
lm(Ct ~ age + target_gene*assay_PCR + id_lab, data)
```

Our goal was to see if adding exogenous data, i.e. C_t values and proportion of positive tests, increased prediction precision.

Models were tuned with the `auto.arima` function, and untuned models were run with $p = 9$, $d = 2$, and $q = 0$ as default parameters, based on the cross-correlation analysis between R_t and C_t time series (Figure S2).

Prediction improvement by models using in addition exogenous data relative to model using past values of R_t only was defined by

$$\Delta\text{MAPE} = \text{MAPE}_{R_t} - \text{MAPE}_{R_t+\text{exo}}$$

S5.4.4 Figure 2 C_t values

To visualize the effect of the different factors, we first perform a linear model to correct for the effect of confounding factors.

We used the general model described above but removed the effect ‘control_variable’, as well as the effects ‘target_gene’, ‘sample_type’, ‘symptoms’ and ‘sampling_facility’ which had little effect and were sometimes lacking for many participants. For each figure, we also removed the main factor of interest (depending on the panel).

The C_t value itself was generated using the `predict.lm` function in R.

S6 Supplementary Figures

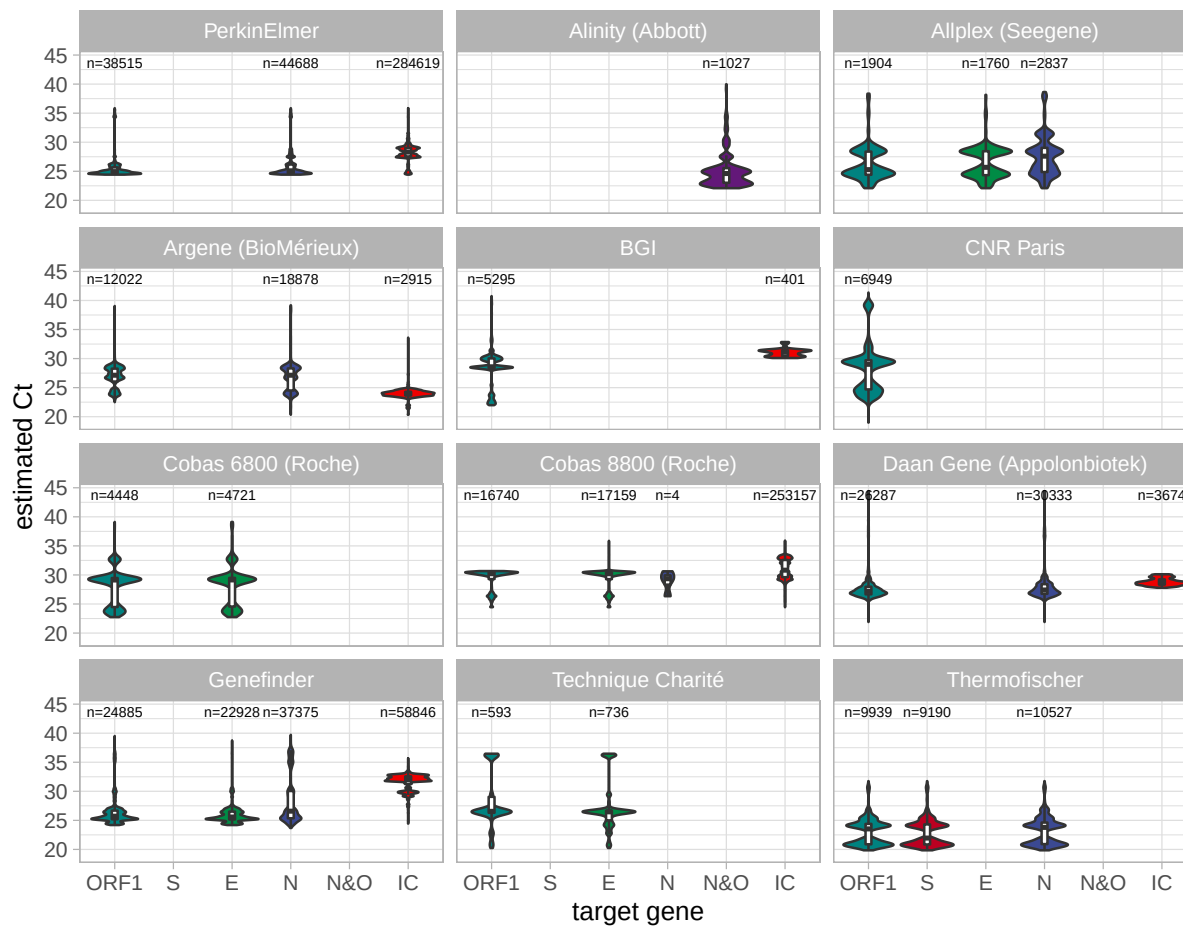


Figure S1: Effect of the RT-PCR assay used on the estimated C_t value as a function of the targeted genomic area. Only tests with at least 1,000 C_t values are shown.

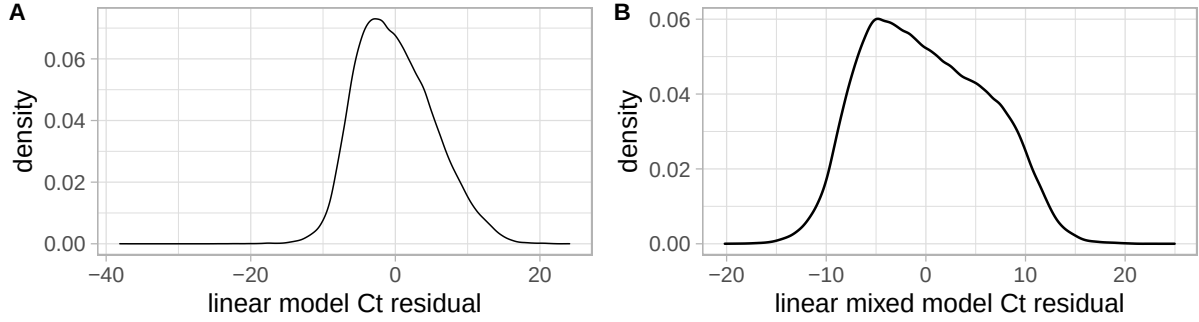


Figure S2: **Distribution of the C_t residuals value.** A) For the linear model used for the main analysis shown in Table 1, B) for the linear model used to generate residuals for the R_t time series analysis.

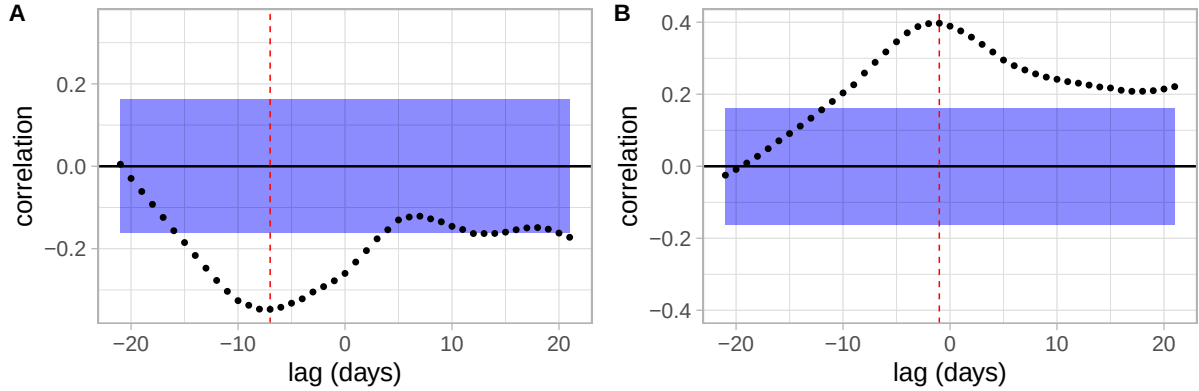


Figure S3: **Cross correlation functions between R_t and A) the median or B) the skewness of the C_t residuals distribution.** The blue shaded areas show the non-significant values (with a 95% threshold) and the red vertical dotted lines the lag with the highest significant correlation. Note that the lag is smaller for the skewness than for the median of the distribution.

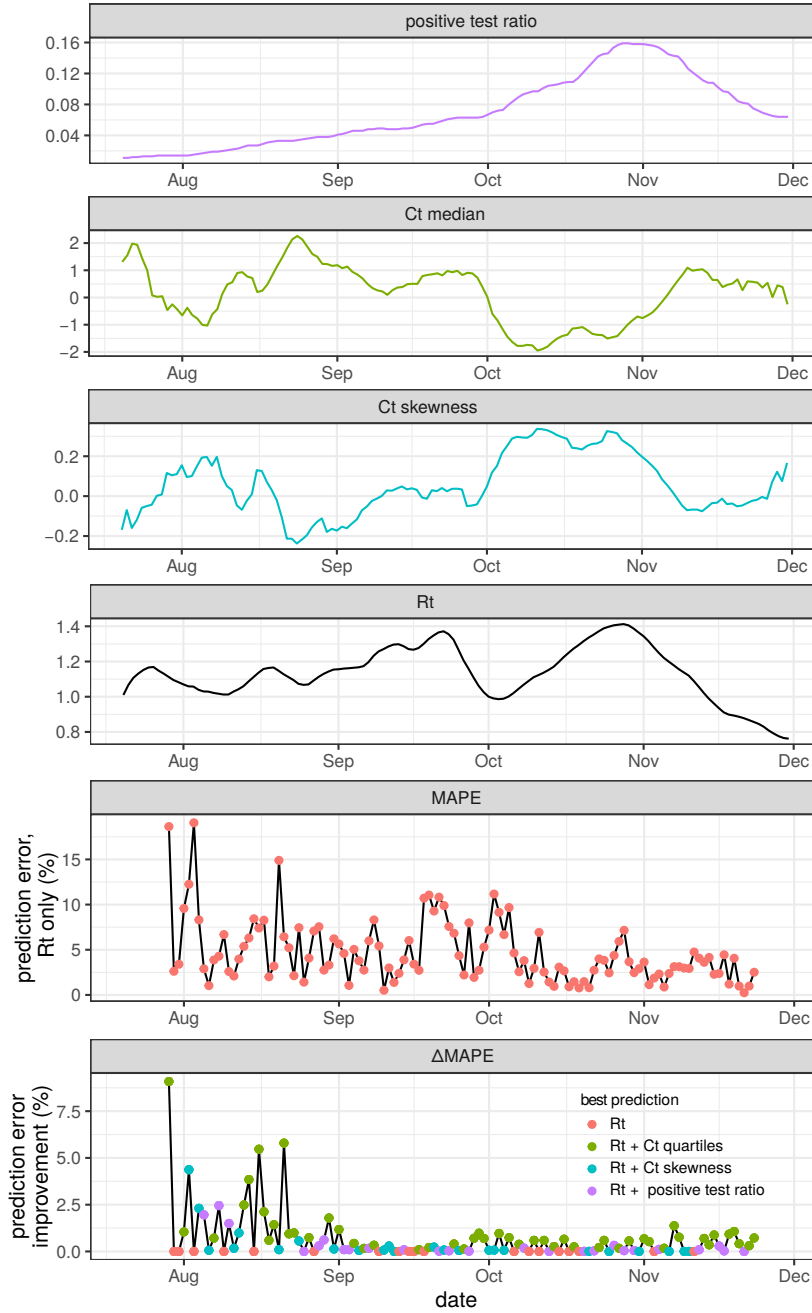


Figure S4: **Predicting temporal reproduction number (R_t) from time series with untuned ARIMA parameters $p = 9, d = 2, q = 0$** The top four panels show the the 7-days rolling averages of the time series of the ratio of positive tests (in purple), the median (in green) and skewness (in cyan) of the daily C_t residuals distribution, and R_t (in black). The bottom panels show the error made by a prediction using only R_t data (red dots) and the potential improvement made by including exogenous data.