Dear Editor,

Gastric cancer represents a remarkable disease burden worldwide, ranking among the first five tumor types in incidence and mortality [1]. Germline DNA variation has been extensively investigated in terms of predisposition to sporadic gastric cancer, which represents more than 90% of all cases [2]. Currently available evidence shows that the fraction of disease burden that can be attributable to known risk polymorphisms is small (< 20%) [2].

Single germline variations of circadian genes (also called clock genes) have been associated with the predisposition of different tumor types [3]. The circadian clock is a time-tracking rhythmic biological system with a periodicity of about 24 hours that enables organisms to anticipate environmental changes and allow them to modify their behavior and physiological functions in the most efficient way. Circadian rhythms are controlled by proteins encoded by circadian genes, which have been discovered in all studied species. Remarkably, the disruption of these rhythms has been linked with risk of different diseases including cancer. In regards to the latter, a growing wealth of evidence supports the potential tumor suppressor role of the biological clock [3, 4].

As the role of circadian gene germline variants has never been explored in the field of gastric cancer susceptibility, with the present work, we intended to test the hypothesis that specific single nucleotide polymorphisms (SNPs) of the circadian genes, such as CLOCK, NPAS2, PER1, PER2, RORA, and TIMELESS, could significantly increase or decrease the predisposition to develop gastric cancer. We considered the 10 SNPs of the above listed 6 circadian genes that are known to be functional or associated with cancer risk or prognosis. The main features of the SNPs are described in our previous study [5].

We conducted a retrospective study based on a total of 1065 subjects comprising of 455 cases of gastric cancer and 610 healthy controls. All of them were of European ancestry. The median age of onset for gastric cancer was 67 years (range, 27-90 years). Among these gastric cancer patients, 249 (54.7%) were males and 206 (45.3%) were females. The median survival was 30.0 months, ranging from 1.0 to 293.0 months. These datasets were already employed in our previous studies [5, 6] and the detailed characteristics of the subjects are summarized in Table 1 and Supplementary Table 1.

Genotyping was performed by real-time PCR. Multivariate logistic regression analysis was performed to assess the associations employing four models of inheritance: allelic, recessive, dominant, and co-dominant. The detailed methods are available in Supplementary information. All the preselected SNPs were successfully genotyped, and no departures from Hardy-Weinberg equilibrium were observed (Supplementary Table 2). The average genotyping success rate of selected SNPs in all participants was 98.9% (range, 96.0%-100%). The mean statistical power for this analysis was 61%. Detailed statistical power for each SNP is reported in Supplementary Table 3.

Associations between the selected circadian genes genetic variations and gastric cancer predisposition were tested assuming 4 models of inheritance. The results are summarized in Table 2. We used odds ratios (ORs) and their corresponding 95% confidence intervals (CI) to measure the strength of association between each polymorphism and gastric cancer susceptibility. Overall, the genetic variants significantly associated with gastric cancer predisposition were: NPAS2 rs895520, PER1 rs3027178, PER2 rs934945, RORA rs339972. In particular, the present analysis suggested that NPAS2 rs895520 minor allele (A) was associated with an increased susceptibility to gastric cancer of 24% under an additive (per allele OR, 1.24; 95% CI, 1.01-1.52; P = 0.036), recessive (OR, 1.56; 95% CI, 1.09-2.24; P = 0.016) and co-dominant (OR, 1.62; 95% CI, 1.07-2.44; P = 0.022) model of inheritance. PER1 rs3027178, a genetic variant with a synonymous functional effect was associated with a reduced predisposition (per allele OR, 0.80; 95% CI, 0.64-0.99; P = 0.037). PER2 rs934945 (C > T) is located on the last exon of PER2

Abbreviations: CI, confidence interval; Ctrl, control; N/A, not applicable; OR, odds ratio; SNP, single nucleotide polymorphism.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2020 The Authors. Cancer Communications published by John Wiley & Sons Australia, Ltd. on behalf of Sun Yat-sen University Cancer Center
Table 1 Characteristics of 455 gastric cancer patients and 610 healthy controls retrospectively included in the present study

| Characteristic          | Gastric cancer patients | Healthy controls |
|-------------------------|-------------------------|------------------|
|                         | n (%)                   | n (%)            |
| Median age (range, years) | 67 (27-90)             | 48 (14-92)       |
| Gender                  |                         |                  |
| Male                    | 249 (54.7)              | 336 (55.2)       |
| Female                  | 206 (45.3)              | 274 (44.8)       |
| Source of controls      |                         |                  |
| Hospital                | N/A                     | 340 (55.7)       |
| Population              | N/A                     | 270 (44.3)       |
| Patient status          |                         |                  |
| Alive                   | 150 (33.0)              | N/A              |
| Dead                    | 305 (67.0)              | N/A              |
| Median survival (range, months) | 30.0 (1.0-293.0) | N/A              |
| Tumor stage             |                         |                  |
| I                       | 131 (28.8)              | N/A              |
| II                      | 84 (18.5)               | N/A              |
| III                     | 109 (24.0)              | N/A              |
| IV                      | 131 (28.8)              | N/A              |

Abbreviation: N/A, not applicable.

locus and has a missense functional effect, leading to the substitution of Glycine-Glutamic acid. Carriers of at least one copy of the minor allele had a decreased predisposition to develop gastric cancer (28%) employing a dominant genetic model (OR, 0.72; 95% CI, 0.53-0.98; \( P = 0.037 \)). Employing a co-dominant model heterozygotes had a 31% risk reduction as compared to homozygotes for the common allele (C) (OR 0.69; 95% CI 0.50-0.94; \( P = 0.019 \)). \( RORA \) rs339972 C allele was associated with a decreased predisposition to develop gastric cancer assuming an additive (per allele OR, 0.78; 95% CI, 0.63-0.98; \( P = 0.032 \)) or dominant (OR, 0.75; 95% CI, 0.56-1.00; \( P = 0.049 \)) genetic model.

To the best of our knowledge, this is the first scientific work investigating the relations between circadian genes DNA genetic variations and the susceptibility to gastric cancer. Therefore, we could not know a priori the genotype-phenotype relation of these SNPs; as a consequence, we tested 4 genetic models of inheritance: allelic, recessive, dominant and co-dominant. When testing the allelic/recessive/dominant models, for those polymorphisms which were significantly associated with the phenotype in more than one model, the best fitting model was considered the one with the lower \( P \) value. Our results indicated that \( NPAS2 \) rs8955520 best-fitted model for the association with gastric cancer was the recessive model of inheritance, while \( RORA \) rs339972 was the allelic model. Interestingly, we found similar results regarding \( NPAS2 \) rs8955520 in our previous work on associations of circadian genes polymorphisms with soft tissue sarcoma susceptibility [5], while there was no difference in terms of \( P \) value for \( RORA \) rs339972 comparing the allelic and the dominant model, nevertheless, both were associated with sarcoma susceptibility as it was for gastric cancer. Since the maximum power was reached when the ‘true’ mode of inheritance of the disease susceptibility loci and the genetic model used in the analysis were concordant [7], it is worth determining the genotype-phenotype relation for each SNP.

We tested the co-dominant model as well, for two reasons: its robust method [7] and its application in testing the circadian genes SNPs associations with different neoplasms [8, 9]. Employing the co-dominant model \( PER2 \) rs934945 heterozygotes had a decreased predisposition compared to homozygotes for the common allele (C) of 31%. Karantanos et al. [9] found no association of \( PER2 \) rs934945 with colorectal cancer neither with the allelic nor with the co-dominant model. Dai and colleagues [8] found no association of \( PER2 \) rs934945 with breast cancer in overall analysis while found a significant association in subgroup analysis. Homozygotes for the minor allele (T) had an increased risk of developing breast cancer only in a specific \( CLOCK \) rs3805151 background (homozygosis for the common allele C). This was in line with the shared idea that genetic variations have different effects in different neoplasms. In particular, this was recently highlighted for prognosis in an interesting work performed by Chang and Lai [4]. They performed a comprehensive study of circadian genes in 21 cancer types that considered genomic, transcriptomic and phenotypic (clinical prognosis) data and they found that circadian genes were substantially altered by somatically acquired deletions and amplifications. Core circadian genes, \( PERs, CRY2, CLOCK, NR1D2, RORA \) and \( RORB \) exhibited global patterns of somatic loss and downregulation across multiple tumor types and that loss-of-function of these genes resulted in increased death risks in patients. However, tumor suppressive qualities appeared to be cancer type-specific. Opposite trend was obtained for bladder and stomach cancers as their “low” loss-of-function of putative tumor-suppressive circadian genes were found to be associated with adverse survival outcomes [4]. In our previous study concerning the associations of gastric cancer prognosis and germline variation of circadian genes [6] we had a similar approach. We found that germline polymorphisms in the circadian pathway were associated with the survival of patients with gastric cancer, independently of established prognostic factors such as disease stage and patient age at diagnosis. In particular, combined information deriving from two SNPs (rs3749474 and rs1801260, two variants of the \( CLOCK \) gene 3’-UTR) allowed us to classify patients into a high or low \( CLOCK \) transcription, with the latter showing a significantly worse prognosis (about 70% increased risk of death). This apparent discrepancy highlights that gastric cancer prognosis and circadian genes relations need further in-depth analysis.
| Gene | SNP        | Genotype | No. of healthy controls | No. of gastric cancer patients | Co-dominant OR (95% CI) | P value | Additive OR (95% CI) | P value | Recessive OR (95% CI) | P value | Dominant OR (95% CI) | P value |
|------|------------|----------|-------------------------|-------------------------------|-------------------------|---------|----------------------|---------|----------------------|---------|----------------------|---------|
| CLOCK | rs1801260  | TT       | 323                     | 236                           | 1.09 (0.80-1.47)         | 0.582   | 0.67 (0.39-1.14)     | 0.136   | 1.20 (0.48-3.00)     | 0.019   | 1.30 (0.97-1.74)     | 0.082   |
|       |            | TC       | 228                     | 183                           | Ref                     | 0.92 (0.74-1.15)         | 0.464   | 0.65 (0.39-1.09)     | 0.100   | 1.00 (0.75-1.33)     | 0.979   |
|       |            | CC       | 56                      | 36                            | Ref                     | 0.92 (0.74-1.15)         | 0.464   | 0.65 (0.39-1.09)     | 0.100   | 1.00 (0.75-1.33)     | 0.979   |
|       |            | Undetermined | 3                       | 0                      | Ref                     | 0.92 (0.74-1.15)         | 0.464   | 0.65 (0.39-1.09)     | 0.100   | 1.00 (0.75-1.33)     | 0.979   |
|       | rs3749474  | CC       | 259                     | 173                           | Ref                     | 1.07 (0.87-1.32)         | 0.522   | 1.05 (0.69-1.60)     | 0.810   | 1.12 (0.83-1.50)     | 0.464   |
|       |            | CT       | 266                     | 206                           | 1.12 (0.82-1.53)         | 0.483   | 1.12 (0.71-1.75)     | 0.627   |                      |         |                      |         |
|       |            | TT       | 83                      | 57                            | Ref                     | 1.12 (0.71-1.75)         | 0.627   |                      |         |                      |         |                      |         |
|       |            | Undetermined | 2                       | 19                      | N/A                     | N/A                  |         |                      |         |                      |         |                      |         |
| NPAS2 | rs895520   | GG       | 211                     | 138                           | 1.06 (0.76-1.47)         | 0.735   | 1.62 (1.07-2.44)     | 0.022   | 1.24 (1.01-1.52)     | 0.036   | 1.56 (1.09-2.24)     | 0.016   |
|       |            | GA       | 294                     | 199                           | Ref                     | 1.06 (0.76-1.47)         | 0.735   | 1.62 (1.07-2.44)     | 0.022   | 1.24 (1.01-1.52)     | 0.036   |
|       |            | AA       | 103                     | 107                           | Ref                     | 1.06 (0.76-1.47)         | 0.735   | 1.62 (1.07-2.44)     | 0.022   | 1.24 (1.01-1.52)     | 0.036   |
|       |            | Undetermined | 2                       | 11                      | N/A                     | N/A                  |         |                      |         |                      |         |                      |         |
|       | rs2305160  | GG       | 283                     | 211                           | 0.95 (0.70-1.28)         | 0.738   | 1.15 (0.70-1.90)     | 0.586   | 1.03 (0.83-1.28)     | 0.807   | 1.18 (0.73-1.91)     | 0.490   |
|       |            | GA       | 264                     | 190                           | 1.06 (0.76-1.47)         | 0.735   | 1.62 (1.07-2.44)     | 0.022   | 1.24 (1.01-1.52)     | 0.036   | 1.56 (1.09-2.24)     | 0.016   |
|       |            | AA       | 59                      | 48                            | Ref                     | 1.06 (0.76-1.47)         | 0.735   | 1.62 (1.07-2.44)     | 0.022   | 1.24 (1.01-1.52)     | 0.036   |
|       |            | Undetermined | 2                       | 11                      | N/A                     | N/A                  |         |                      |         |                      |         |                      |         |
| PER1  | rs3027178  | TT       | 281                     | 226                           | Ref                     | 0.80 (0.59-1.08)         | 0.141   | 0.63 (0.39-1.02)     | 0.062   | 0.80 (0.64-0.99)     | 0.037   | 0.70 (0.44-1.11)     | 0.132   |
|       |            | TG       | 253                     | 185                           | Ref                     | 0.80 (0.59-1.08)         | 0.141   | 0.63 (0.39-1.02)     | 0.062   | 0.80 (0.64-0.99)     | 0.037   | 0.70 (0.44-1.11)     | 0.132   |
|       |            | GG       | 76                      | 44                            | Ref                     | 0.80 (0.59-1.08)         | 0.141   | 0.63 (0.39-1.02)     | 0.062   | 0.80 (0.64-0.99)     | 0.037   | 0.70 (0.44-1.11)     | 0.132   |
| PER2  | rs934945   | CC       | 386                     | 314                           | 0.69 (0.50-0.94)         | 0.199   | 1.20 (0.48-3.00)     | 0.704   | 0.79 (0.60-1.04)     | 0.087   | 1.34 (0.54-3.34)     | 0.530   |
|       |            | CT       | 206                     | 129                           | Ref                     | 0.69 (0.50-0.94)         | 0.199   | 1.20 (0.48-3.00)     | 0.704   | 0.79 (0.60-1.04)     | 0.087   | 1.34 (0.54-3.34)     | 0.530   |
|       |            | TT       | 17                      | 12                            | Ref                     | 0.69 (0.50-0.94)         | 0.199   | 1.20 (0.48-3.00)     | 0.704   | 0.79 (0.60-1.04)     | 0.087   | 1.34 (0.54-3.34)     | 0.530   |
|       |            | Undetermined | 1                       | 0                      | N/A                     | N/A                  |         |                      |         |                      |         |                      |         |                      |         |
| RORA  | rs339972   | TT       | 358                     | 230                           | 1.35 (1.00-1.83)         | 0.053   | 0.98 (0.51-1.86)     | 0.940   | 1.17 (0.92-1.48)     | 0.210   | 0.86 (0.46-1.62)     | 0.640   |
|       |            | TG       | 213                     | 184                           | 1.35 (1.00-1.83)         | 0.053   | 0.98 (0.51-1.86)     | 0.940   | 1.17 (0.92-1.48)     | 0.210   | 0.86 (0.46-1.62)     | 0.640   |
|       |            | GG       | 38                      | 24                            | Ref                     | 0.78 (0.58-1.06)         | 0.118   | 0.62 (0.37-1.04)     | 0.073   | 0.78 (0.58-1.06)     | 0.118   | 0.62 (0.37-1.04)     | 0.073   |
|       |            | Undetermined | 1                       | 17                      | N/A                     | N/A                  |         |                      |         |                      |         |                      |         |                      |         | (Continues)
Moreover, we could not replicate the data reported by Qu and colleagues [10] on the association between PER variants and prognosis. Different ethnicity (European vs. Asian), sample size (the Asian series was more than two-fold larger) and disease stage composition (only our study included patients with advanced and metastatic gastric cancer) might partly explain this discrepancy. Nevertheless, differences were found by 2 groups studying PER2 expression as a prognostic factor for gastric cancer in patients with Asian ethnicity. Zhao and colleagues [11] found that PER2 expression was downregulated in most gastric cancer tissues, while Hu and colleagues [12] found that it was upregulated.

To our knowledge, this is the first analysis investigating the hypothesis of an association between germline genetic variations of the circadian pathway with gastric cancer susceptibility. The power of our study is not optimal, and the present study should be considered as a pilot work that warrants further validation in different datasets. Nevertheless, our results showed that the 4 circadian clock variants were clinically and statistically associated with gastric cancer predisposition.

### DECLARATIONS

#### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of Padova University Hospital (identifier: prot#448). Written informed consent was obtained from all patients.

#### CONSENT FOR PUBLICATION

Not applicable.

#### AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article and its additional files.

#### COMPETING INTERESTS

The authors declare that they have no competing interests.

#### FUNDING

This work was supported by the University of Padova (DOR1944742).

### AUTHORS’ CONTRIBUTIONS

C.B. and S.M.: analyzed data and co-wrote the manuscript. S.R.: performed experiments. A.M.: managed clinicopathological data. D.N.: provided critical revision of the manuscript. All authors read and approved the final manuscript.

### ACKNOWLEDGEMENTS

We thank the personnel of the Biobank of the First Surgical Clinic (Padova University Hospital, Padua, Italy) and particular Dr. Andrea Ferron (Padova University Hospital, Padua,
Italy) for organizing the sampling activity and Dr. Enrico Lion (Padova University Hospital, Padua, Italy) for organizing the informed consent retrieval.

Senthilkumar Rajendran¹,²*, Clara Benna¹,²*, Alberto Marchet³
Donato Nitti¹,²
Simone Mocellin¹,⁴

¹Department of Surgery Oncology and Gastroenterology, University of Padova, Padua 35128, Italy
²First Surgical Clinic, Padova University Hospital, Padua 35128, Italy
³Multidisciplinary Day-surgery Unit, Padova University Hospital, Padua 35128, Italy
⁴Surgical Oncology Unit, Veneto Institute of Oncology IOV-IRCCS, Padua 35128, Italy

Correspondence
Clara Benna, Department of Surgery Oncology and Gastroenterology, University of Padova, Padua 35128, Italy and First Surgical Clinic, Padova University Hospital, Padua 35128, Italy.
Email: clara.benna@unipd.it

*These authors contributed equally to this work.

REFERENCES
1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin. 2019;69:7–34.
2. Mocellin S, Verdi D, Pooley KA, Nitti D. Genetic variation and gastric cancer risk: a field synopsis and meta-analysis. Gut. 2015;64:1209–19.
3. Benna C, Helfrich-Forster C, Rajendran S, Monticelli H, Pilati P, Nitti D, et al. Genetic variation of clock genes and cancer risk: a field synopsis and meta-analysis. Oncotarget. 2017;8:23978–95.
4. Chang WH, Lai AG. Timing gone awry: distinct tumour suppressive and oncogenic roles of the circadian clock and crosstalk with hypoxia signalling in diverse malignancies. J Transl Med. 2019;17:019-1880-9.
5. Benna C, Rajendran S, Spiro G, Tropea S, Del Fiore P, Rossi CR, et al. Associations of clock genes polymorphisms with soft tissue sarcoma susceptibility and prognosis. J Transl Med. 2018;16:338,018-1715-0.
6. Rajendran S, Benna C, Monticelli H, Spiro G, Menin C, Mocellin S. Germline variation of circadian pathway genes and prognosis of gastric cancer patients. Gut. 2018;67:779–80.
7. Lettre G, Lange C, Hirschhorn JN. Genetic model testing and statistical power in population-based association studies of quantitative traits. Genet Epidemiol. 2007;31:358–62.
8. Dai H, Zhang L, Cao M, Song F, Zheng H, Zhu X, et al. The role of polymorphisms in circadian pathway genes in breast tumorigenesis. Breast Cancer Res Treat. 2011;127:531–40.
9. Karantanos T, Theodoropoulos G, Gazouli M, Vaiopoulos A, Karantanou C, Stravopodis DJ, et al. Association of the clock genes polymorphisms with colorectal cancer susceptibility. J Surg Oncol. 2013;108:563–7.
10. Qu F, Qiao Q, Wang N, Ji G, Zhao H, He L, et al. Genetic polymorphisms in circadian negative feedback regulation genes predict overall survival and response to chemotherapy in gastric cancer patients. Sci Rep. 2016;6:22424.
11. Zhao H, Zeng ZL, Yang J, Jin Y, Qiu MZ, Hu XY, et al. Prognostic relevance of Period1 (Per1) and Period2 (Per2) expression in human gastric cancer. Int J Clin Exp Pathol. 2014;7:619–30.
12. Hu ML, Yeh KT, Lin PM, Hsu CM, Hsiao HH, Liu YC, et al. Deregulated expression of circadian clock genes in gastric cancer. BMC Gastroenterol. 2014;14:67, 230X-14-67.

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