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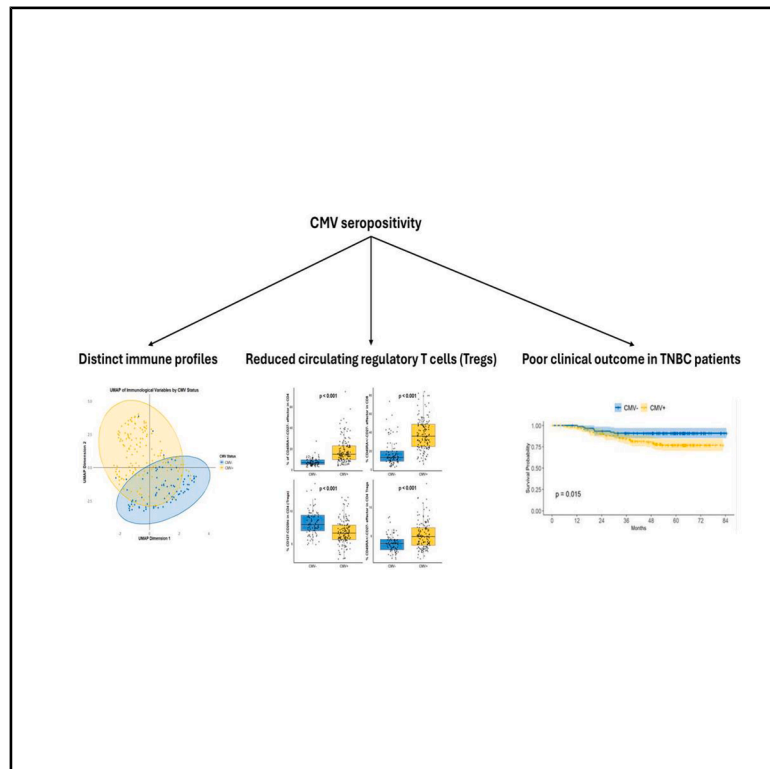
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CMV seropositivity associates with poor clinical outcome in triple negative breast cancer

Graphical abstract



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In brief

Cell biology; Oncology; Pathology

Highlights

- CMV infection shapes the immune system, but its impact on breast cancer is unknown
- CMV seropositivity is associated with worse survival in patients with TNBC
- CMV seropositivity is associated with decreased frequency of regulatory T cells
- CMV serology could help risk stratification to adapt treatment for a better outcome



Article

CMV seropositivity associates with poor clinical outcome in triple negative breast cancer

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SUMMARY

Cytomegalovirus (CMV) infection modifies immune fitness and T cell subset distribution in healthy donors. We analyzed a recent prospective cohort of 240 patients bearing triple-negative breast cancer for CMV serology status and T cell subsets and assessed recurrence-free survival (RFS) and overall survival (OS). CMV seropositivity was significantly associated with worse RFS (HR = 2.6, 95%CI = 1.2–5.7) and OS (HR = 3.0, 95%CI = 1.2–7.4) in multivariable analyses. A prognostic model incorporating CMV status alongside tumor and nodal stage improved risk stratification. The impact of CMV status was not confirmed in another less well characterized, more ancient cohort ($n = 507$), probably related to the different therapeutic strategies used in the early 2000s. CMV status had a strong impact on T cell subset distribution with increased frequency of effector CD8 T cells and decreased proportion of Tregs. The impact of CMV infection on cancer patient survival remains to be determined during chemo-immunotherapy, which is now the standard of care.

INTRODUCTION

Breast cancer (BC) is the most frequent cancer and the second leading cause of death by cancer in women worldwide.¹ Triple negative breast cancer (TNBC), accounts for 15–20% of breast cancers,² and is associated with a worse prognosis than other BC molecular subtypes and often occurs in younger women. TNBC is characterized by a lack of estrogen receptors (ERs),

progesterone receptors (PRs), and human epidermal growth factor receptor 2 (HER2) expression by immunostaining. It is a biologically aggressive tumor, characterized by moderate/high-grade and highly proliferative cancer cells, which, together with limited treatment options, leads to the poorest prognosis among BC subtypes. The standard treatment for early TNBC combines neo-adjuvant or adjuvant chemotherapy, radiotherapy, and surgery, and, more recently addition of immune checkpoint



inhibitors (ICIs). However, treatment relapses are frequent, and new biomarkers are needed to help identify the patients most at risk of recurrence, who may benefit from treatment intensification.

TNBC is the most immune-activated subtype among all breast cancers, as evidenced by higher levels of tumor-infiltrating lymphocytes (TILs)³ and the effectiveness of ICI in both the early and metastatic setting.^{4–6} Increasing levels of TILs correlate linearly with decreased risk for recurrence or death,⁷ as well as with treatment efficacy, as measured by the pathological complete response rate after neoadjuvant chemotherapy (NAC). These data suggest a role for the immune system in the efficacy of NAC, as also suggested by the correlation between the increased frequency of effector CD4 T cells during NAC and tumor regression.⁸ The response to NAC in a given patient could be related either to intrinsic factors of the tumor cells or to the fitness of the immune system. Genetic and environmental factors modulating the inter-individual variability of circulating immune cell subsets have been extensively studied in healthy individuals.⁹ In particular, a history of infection with the cytomegalovirus (CMV) is a major factor in the immune cell subset variance.^{9,10} CMV is a herpes virus that infects ~50% of humans in France, with important geographical variations worldwide, and persists in a latent state for life, with some asymptomatic reactivation from time to time. CMV is acquired during infancy, but also throughout adulthood.¹¹ Several studies have reported CMV presence in breast cancer tissues,¹² and the virus has been shown to transform normal mammary cells *in vitro* and establish TNBC-like breast cancer in mice models.^{13,14} Additionally, in all individuals, encounters with CMV shape the immune system, and particularly the T cell compartment, with a 4- to 12-fold expansion in the number of effector memory T cells circulating in the blood. Multiple studies have highlighted the important impact of CMV seropositivity on the overall immune system fitness,¹⁵ with CMV seropositivity being associated with increased response to influenza,¹⁶ poor response to vaccination,^{17,18} increased atherosclerosis,¹⁹ and decreased survival in glioblastoma.²⁰ CMV infection also impacts SARS-Cov2 acute viral infection, with CMV seropositivity leading to a 2-fold increased risk of being hospitalized.¹⁵ Finally, CMV seropositivity is also associated with better outcomes after immune checkpoint blockade treatment, with a prolonged overall survival in patients with metastatic melanoma treated with anti-PD1 - but not with anti-CTLA4 plus anti-PD1, as well as a lower occurrence of immune-related adverse events.²¹

In this work, we hypothesized that factors that modulate immune system fitness, and particularly the T cell compartment, may have an impact on TNBC outcomes. This led us to study whether prior CMV infection would impact the prognosis of patients with TNBC. To test this, we analyzed a large ($n = 240$) recent prospective cohort of patients with TNBC with available CMV serology at baseline and compared clinical outcomes over a 5-year follow-up according to CMV serology status. We also analyzed a less well characterized and older (>19 years follow-up) cohort ($n = 507$) for which a limited number of clinical characteristics could be retrieved, and CMV status was characterized on biobanked serums.

RESULTS

Patient characteristics

Two hundred and forty patients were analyzed in the prospective study corresponding to the “recent” cohort (Figure 1). Table 1 summarizes the clinicopathological characteristics of the 240 patients. The median age was 50 years (range, 24–88). All patients underwent breast surgery, including 157 patients (65%) who received neoadjuvant chemotherapy (NAC) and 88 patients (37%) who received adjuvant chemotherapy. Most patients (95%) received adjuvant external beam radiation therapy.

Among the 240 patients, 150 patients had detectable IgG directed against CMV (62.5% seropositive patients), and 90 patients did not. The level of IgGs was reaching the higher limit in most patients, which did not allow titers comparison. Given the low rate of yearly seroconversion in France,⁹ IgM directed against CMV was not measured in our study. There was no difference in demographics or treatment between patients with CMV seropositive and seronegative (Table 1). After a median follow-up of 4.8 years, 40 patients (17%) developed tumor recurrence, and 33 patients (14%) died during the follow-up period.

Association with outcomes

Cytomegalovirus seropositivity negatively impacts the overall survival and relapse-free survival in triple negative breast cancer, independently of other associated cofactors

In the NAC sub-group (65% of patients), the pCR rate was similar between patients with CMV+ and CMV- (48% and 39%, respectively, $p = 0.21$). We further investigated the ability of CMV status and classical clinicopathological factors to impact survival in the whole cohort. The results of univariable and multivariable Cox regression models are presented in Table 2. In univariable analysis, advanced tumor stage (T3 and T4), lymph node involvement (N+), and CMV+ were associated with a significantly higher risk of recurrence and death (Figure 2). In multivariable analysis for RFS (Table 2), lymph node involvement (HR 2.3, 95% CI 1.2–4.4) and CMV+ (HR 2.6, 95% CI 1.2–5.7) remained independent statistically significant prognostic factors. In multivariable analysis for OS (Table 2), advanced tumor stage (HR 2.2, 95% CI 1.0–4.6), lymph node involvement (HR 2.3, 95% CI 1.1–4.8), and CMV+ (HR 3.0, 95% CI 1.2–7.4) remained independent statistically significant prognostic factors.

Prognosis score for risk stratification

A prognosis score integrating cytomegalovirus serology improves the identification of high-risk patients at baseline

Given that T stage, N stage, and CMV status were significant prognostic factors for RFS and OS in univariate analyses, we developed a prognostic scoring system based on three binary factors: tumor stage (T1-T2 vs. T3-T4), nodal status (N+ vs. N-), and CMV status (CMV+ vs. CMV-). This prognostic score divided the patients into four prognostic groups: excellent (T1-T2, N-, and CMV-: $n = 57$, 24%), good (presence of any single risk factor including T3-T4, N+, or CMV+: $n = 114$, 47%), intermediate (presence of two risk factors including T3-T4 and N+, T3-T4 and CMV+, or N+ and CMV+: $n = 53$, 22%) and poor (presence of

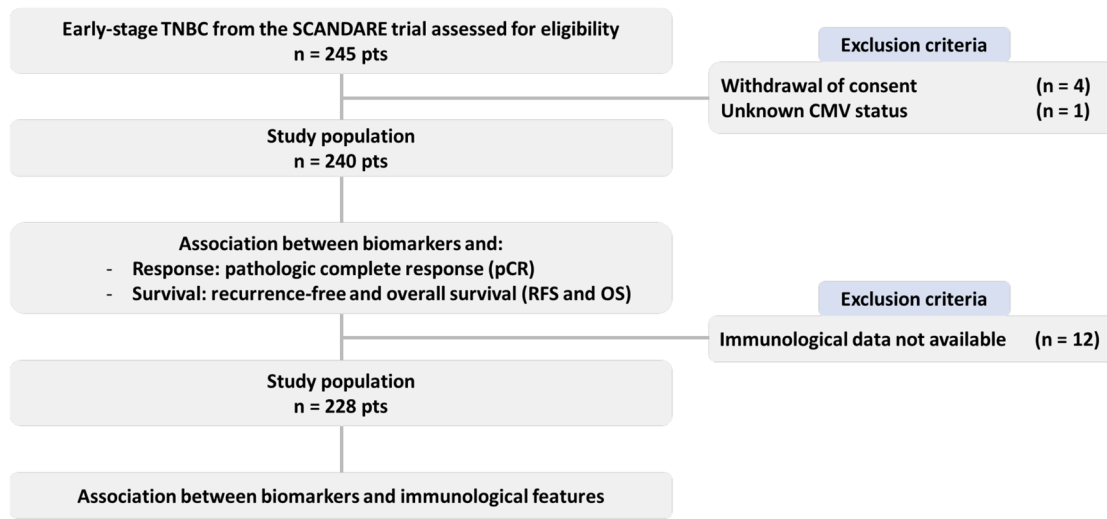


Figure 1. Patient characteristics in the recent cohort
Flow-chart.

all three risk factors including T3-T4, N+, and CMV+: $n = 16$, 7%). This score accurately stratified the population into four prognostic sub-groups ($p < 0.001$ both for mRFS and mOS) (Figure 3). At 5 years, the rate of RFS:OS was approximately 98%:98% for the excellent prognostic group, 83%:89% for the good prognostic group, 74%:79% for the intermediate prognostic group, and 57%:64% for the poor prognostic group ($p < 0.001$ both for RFS and OS; Figure 2). We verified the interest in including CMV serostatus in this prognosis score (Table 3). CMV+ serostatus added significant prognostic values to the multivariable model obtained with the backward elimination process for RFS and OS ($p = 0.008$ and $p = 0.007$, respectively).

Associations with immunological data Cytomegalovirus seropositivity associates with a lower amount of circulating regulatory T cells

As CMV status considerably impacts T cell subset distribution in healthy subjects,⁹ we analyzed whether this was also the case in patients with TNBC. Using peripheral blood drawn at baseline, we compared the proportions of circulating immune subsets in CMV seropositive and seronegative patients using a 15 antibody panel. In peripheral blood from the patients enrolled in the cohort, we compared the proportions of circulating immune subsets in CMV seropositive and seronegative patients. We first defined 36 immune phenotypes arising from the analysis of our 15-parameter T cell panel (Figure S1; Table S1). The 36-dimensional dataset was reduced to two dimensions using Uniform Manifold Approximation and Projection (Figure 4A). Most patients projected on different regions of the UMAP according to their CMV status, confirming a strong impact of CMV infection on circulating immune T cell subsets in patients with TNBC before treatment.

To determine which immune subsets were different between patients with CMV^{neg} and CMV⁺, we then compared the proportions of the 36 immune phenotypes among CMV seropositive and seronegative patients (Figure S2; Tables S1 and S2). These

immune phenotypes correspond to different T lymphocyte subsets, including states of differentiation for CD4 and CD8 T cells, as well as regulatory T cells and PD1 checkpoint inhibitor expression. As observed in healthy populations,⁹ CMV seropositivity was associated with the increased proportion of effector (CD27^{neg}CD45RA^{+/-}) in the CD4⁺ T cells (effect size 2.3; $P < 2e^{-16}$) and to a higher extent, of the proportion of effector (CD27^{neg}CD45RA^{+/-}) CD8⁺ T cells (effect size 2.8, $P < 2e^{-16}$) (Figure 4B). Interestingly, in the TNBC cohort, CMV seropositivity was also associated with lower proportions of Tregs (CD25^{hi}CD127^{lo}) in the CD4⁺ T cells circulating in the blood ($p = 7.3e^{-8}$) and with a higher proportion of effector Tregs (CD27⁻CD45RA⁺) in the Tregs (CD25^{hi}CD127^{lo} CD4⁺) ($P = 7e^{-8}$), *i.e.*, Tregs undergoing chronic stimulation. Thus, both the effector and regulatory arms of the immune response were impacted by the CMV infection (Figure 4B). This chronic stimulation may dilute the specific immune response against the tumor.

Finally, on 80 patients where quantitative anti-CMV IgG titers were available, we further evaluated their association with the immunological parameters evaluated in our study (Table S3). anti-CMV IgG titers were normally distributed (Figure 4C), and positively associated with ICOS⁺ conventional CD4 T cells (Figure 4D). ICOS is a T cell coregulatory receptor that provides a costimulatory signal to T cells during antigen-mediated activation and may increase help for B cells. Higher levels of this population could indicate increased overall activation. In contrast, there was no association with Treg proportions.

Impact of cytomegalovirus status on the survival of patients treated for triple negative breast cancer in the early 2000s

To assess the generality of the previous findings, we assessed the CMV status at the diagnosis of 507 patients treated for TNBC at Institut Curie in the early 2000s, for which minimal clinical information was available (Table S4). Notably, the proportion of patients treated by NAC was much lower (21%) in the old

Table 1. Patient characteristics in the recent cohort

	All <i>n</i> = 240 pts	CMV+ <i>n</i> = 150 pts	CMV- <i>n</i> = 90 pts	<i>p</i> value
Median [range], <i>n</i> (%)				
Clinicopathological characteristics				
Demographic parameters				
Age (years)	50 [24–88]	51 [24–83]	49 [25–88]	0.22
BMI (kg/m ²)	23.8 [16.5–42.9]	24.2 [16.5–39.9]	22.9 [18.1–42.9]	0.08
Post-menopausal status	124 (52)	81 (54)	43 (48)	0.35
TNM Stage (AJCC classification)				
T1-2/T3-4	201 (84)/39 (16)	121 (81)/29 (19)	80 (89)/10 (11)	0.10
N+	79 (33)	47 (31)	32 (36)	0.08
Histologic parameters				
Estrogen-receptor (%)	0 [0–5]	0 [0–0]	0 [0–5]	0.60
Progesterone-receptor (%)	0 [0–5]	0 [0–0]	0 [0–5]	0.49
HER2-0/HER2-low	189 (78)/52 (22)	118 (79)/32 (21)	70 (78)/20 (22)	0.87
Histologic grade I-II/III	67 (28)/173 (72)	44 (29)/106 (71)	23 (26)/67 (74)	0.59
Ki67 (%)	60 [5–98]	60 [5–98]	60 [12–94]	0.76
Molecular alterations				
BRCA1-2 mutations	29 (12)	14 (9)	15 (17)	0.09
Treatment				
Surgery	240 (100)	150 (100)	90 (100)	1.00
Neo-adjuvant chemotherapy	157 (65)	100 (67)	57 (63)	0.36
pCR	70 (45)	48 (48)	22 (39)	0.21
Adjuvant therapy				
Chemotherapy	88 (37)	55 (37)	33 (37)	1.00
Radiotherapy	228 (95)	141 (94)	89 (99)	0.05
Outcomes				
Recurrence	40 (17)	32 (21)	8 (9)	<0.01
Death	33 (14)	27 (18)	6 (7)	0.01

Bold indicates *p* value < 0.05.

cohort than in the recent one (65%) (Figure 5A), reflecting the change in therapeutic practice. To check for data quality, we first verified that nodal involvement was indicative of poor prognosis in the old cohort (Figures S4A and S4B) as it was in the recent cohort (Figure 2D). RFS could not be assessed in this cohort because of a lack of reliable data. Although not statistically significant, the OS was slightly better in the recent versus the old

cohort (Figure 5B). A similar trend was observed for the NAC or primary surgery groups (Figures 5C and 5D).

CMV status did not impact OS of the old cohort (Figure 6A). A small trend for the worst OS in patients with CMV+ was observed for the patients of the old cohort treated by NAC, but did not reach statistical significance, probably because of the small number (*n* = 71) of patients in this category (Figure 6B). No

Table 2. Outcomes analysis using Cox models for recurrence-free survival (RFS) and overall survival (OS) for the whole cohort (*n* = 240 patients)

<i>n</i> = 240 pts	Recurrence-Free Survival				Overall Survival			
	Univariate		Multivariate		Univariate		Multivariate	
Variable	HR (CI 95%)	<i>p</i> value	HR (CI 95%)	<i>p</i> value	HR (CI 95%)	<i>p</i> value	HR (CI 95%)	<i>p</i> value
Age <50 years (vs. ≥ 50)	0.8 (0.4–1.5)	0.45	–	–	0.9 (0.4–1.7)	0.66	–	–
T stage III-IV (vs. I-II)	2.6 (1.3–5.2)	<0.01	1.8 (0.9–3.8)	0.09	3.1 (1.5–6.4)	<0.01	2.2 (1.0–4.6)	0.04
N+ (vs. N-)	2.5 (1.3–4.7)	<0.01	2.3 (1.2–4.4)	0.02	2.6 (1.3–5.2)	<0.01	2.3 (1.1–4.8)	0.02
HER2-low (vs. HER2-zero)	1.1 (0.5–2.4)	0.76	–	–	1.7 (0.8–3.6)	0.15	–	–
Histologic grade 3 (vs. 2)	0.8 (0.4–1.5)	0.41	–	–	0.7 (0.3–1.4)	0.37	–	–
Ki67 > 60% (vs. ≤ 60%)	0.8 (0.4–1.6)	0.59	–	–	0.8 (0.4–1.6)	0.55	–	–
CMV + (vs. CMV-)	2.5 (1.2–5.5)	0.01	2.6 (1.2–5.7)	0.02	2.9 (1.2–7.1)	0.02	3.0 (1.2–7.4)	0.01

Bold indicates *p* value < 0.05.

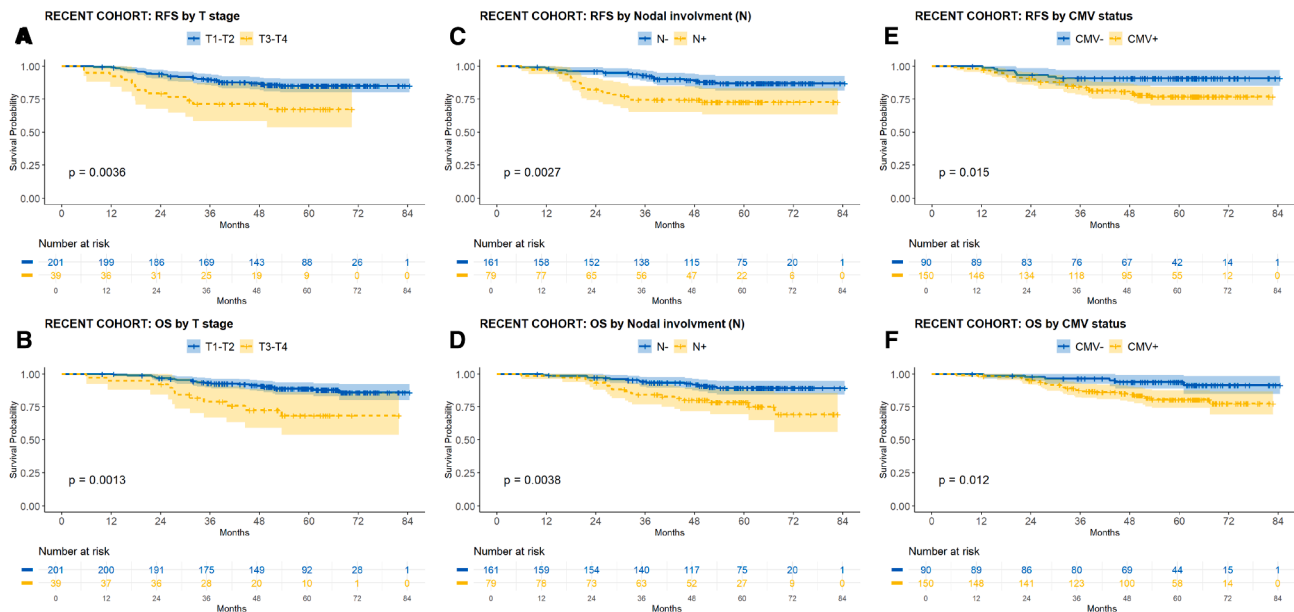


Figure 2. CMV seropositivity negatively impacts the overall survival and relapse-free survival in TNBC, independently of other associated cofactors

KM curves for recurrence-free survival (RFS) (A, C, and E) and overall survival (OS) (B, D, and F), according to T stage (A and B), Nodal involvement (C and D), and CMV status (E and F) for the whole cohort ($n = 240$ patients).

impact of CMV status was observed in the old cohort of patients treated by primary surgery (Figure 6C) while a trend for worse prognosis in patients with CMV+ was observed for primary surgery in the recent cohort. Altogether, these results suggest that the better OS of patients with CMVneg observed in the recent cohort is probably related to improved therapeutic modalities with a particular role for NAC.

DISCUSSION

Here, using a large cohort with a 5-year follow-up, we describe the added value of CMV serology as a new biomarker to help identify the patients with triple negative breast cancer (TNBCs) who are most at risk of relapses. Combined with traditional T-stage and Nodal involvement factors, CMV serostatus better defines a subgroup of patients with poor clinical outcome, and as such, in need for intensification treatment. CMV serology is easy to assess, reproducible, and affordable, and could be a relevant addition to help clinicians in their routine practice.

Of note, we did not measure anti-CMV IgM levels in our study since a single determination cannot efficiently monitor recent infection or reactivation.²² Although it is possible that some of our patients did experience these viral events in the course of the 4.8 years median follow-up, this would potentially reclassify a maximum of 1–3 case among the 90 seronegative patients (rate of seroconversion in France: 1–2%⁹), and this would not modify our results of an association of CMV seropositivity with TNBC relapses.

The health impact of CMV chronic infection, which happens in about 50% of the population worldwide, is being increasingly described.²³ In the current study, we extend this observation

to the impact of CMV seropositivity on patients with TNBC. The mechanisms underlying this observation are unclear. Briefly, a direct mechanism, where the CMV virus itself leads to malignant transformation and cancerogenesis, may be involved as previously described.^{13,14,24} Alternatively, given the strong impact of CMV on the circulating immune compartment,^{9,25,26} the mechanism could be indirect: CMV infection could perturb the overall immune system to decrease the immune response toward breast cancer cells, thereby impacting the evolution of breast cancer in return. In our study, we did not address the question of the virus itself and/or different strains in the tumors. Instead, we tackled the second hypothesis by performing a standardized immuno-monitoring on the peripheral blood of all patients from the recent cohort. Of note, our choice of antibody markers did not allow the tracking of some other populations potentially impacted by CMV infection, such as NKG2C+ NK cells. We observed a strong impact of CMV serostatus on the distribution of T cell subsets according to their differentiation, with a 2-to-4-fold increase in effector T cells circulating in CMV seropositive patients. This effect is not specific to TNBC, as it has been observed in healthy cohorts⁹ and also in uveal melanoma.²⁷ Although CMV viral reactivation may potentially be more frequent in patients with TNBC,²⁸ our results show that the related expansion of effector T cells is comparable in TNBC versus healthy patients. Interestingly, our results also evidence a decreased proportion of regulatory T cells (Tregs) in the blood of patients with CMV seropositive TNBC, as also observed in patients with metastatic melanoma.²¹ This is not a typical association, as CMV was found to have no impact on the amount of circulating regulatory T cells in a 1000 healthy individuals cohort.⁹ It is therefore unlikely that the low frequency of Tregs

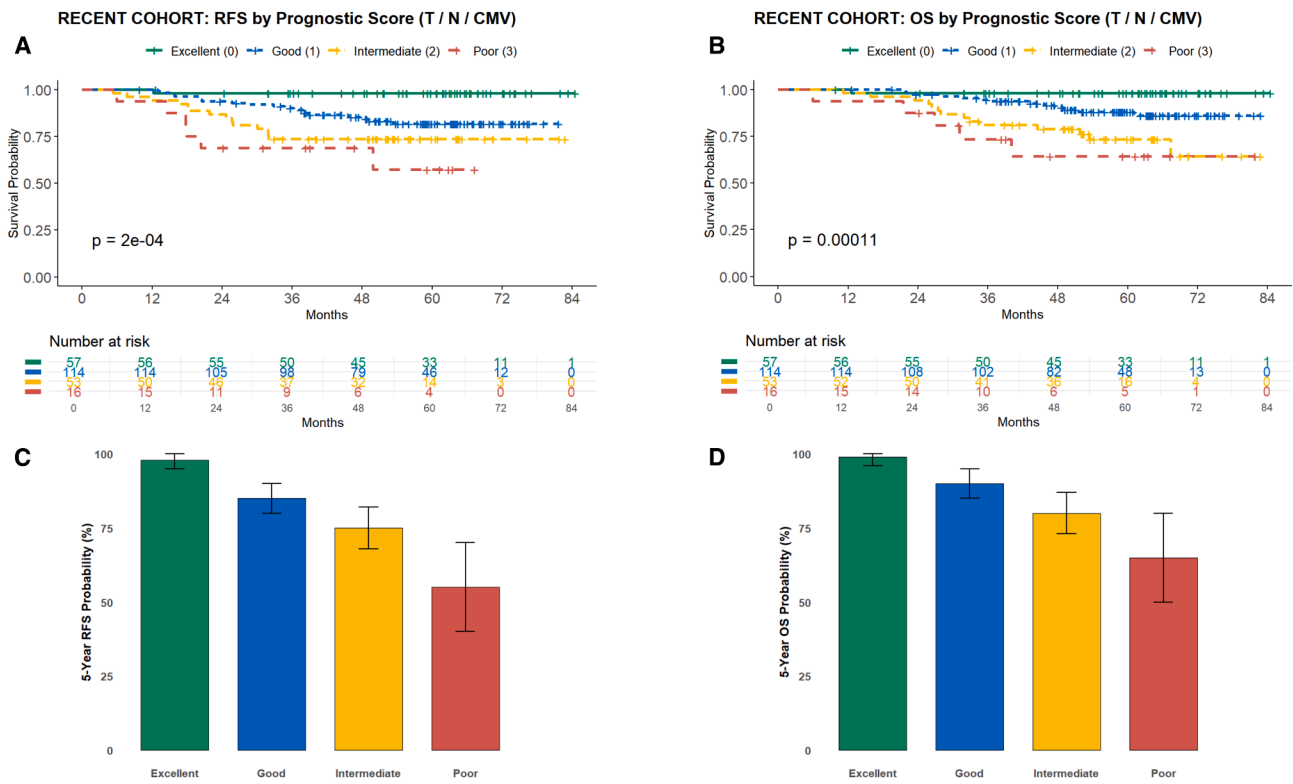


Figure 3. A prognosis score integrating CMV serology improves the identification of high-risk patients at baseline

KM curves for recurrence-free survival (RFS) (A) and overall survival (OS) (B) according to the prognostic score (T-stage, Nodal involvement, and CMV status) for the whole cohort ($n = 240$ patients). Five-year recurrence-free survival (5years-RFS) (C) and five-year overall survival (5years-OS) (D) probability (%) according to risk group (mean \pm SD). Definition of risk groups: **Excellent in green** (T1–T2, N–, and CMV–), **Good in blue** (presence of any single risk factor: T3–T4, N+, or CMV+), **Intermediate in yellow** (presence of two risk factors: T3–T4 and N+, T3–T4 and CMV+, or N+ and CMV+), and **Poor in red** (presence of all three risk factors: T3–T4, N+, and CMV+).

in patients with CMV seropositive TNBC is due to a dilution by the increased effector cells. This could potentially reflect the increased reactivation of the CMV virus in tumors, with concomitant local increase in effector Tregs. As the abundance of Tregs in the tumor bed seems to be an important predictive biomarker for TNBC,²⁹ it will be interesting to further evaluate the intra-tumoral infiltration of Tregs in TNBC tumors in CMV seropositive and seronegative patients, together with other recognized participants of the tumor microenvironment such as tumor-associated macrophages.

Notably, CMV status had no impact on OS of patients with TNBC treated 20–25 years ago in the same center. In that old cohort, NAC

was much less used than in recent years, leading the primary surgery group in the recent cohort to encompass mostly small tumors without nodal involvement. Accordingly, OS was excellent in this group (Figure 6C). The lack of statistically significant impact of the CMV serostatus on OS in both the recent cohort treated by primary surgery and the old cohort treated by NAC may be due to the low number of patients in these two categories.

In contrast to the patients with metastatic melanoma treated by anti-PD1,²¹ in our TNBC cohort treated by radio-chemotherapy, CMV seropositivity was associated with the worst prognosis. Although the reason for this discrepancy is unclear, it is possible that the type of treatment significantly impacts this

Table 3. Tests for the additional prognostic value of CMV status

Cox model variables	Recurrence-Free Survival		Overall Survival	
	LR χ^2	LR p value	LR χ^2	LR p value
Multivariable model <u>with</u> CMV status	18.3	–	19.2	–
Multivariable model <u>without</u> CMV status	11.3	0.008	11.9	0.007

Likelihood ratio test and p values were generated comparing multivariable Cox proportional hazard models with and without the addition of CMV status (positive versus negative) to test its additional prognostic value. The multivariable Cox model was generated with backward elimination for Cox regression. The multivariable model included the following clinical variables: T stage (III–IV versus I–II), lymph node involvement (N+ versus N–), and CMV status (positive versus negative).

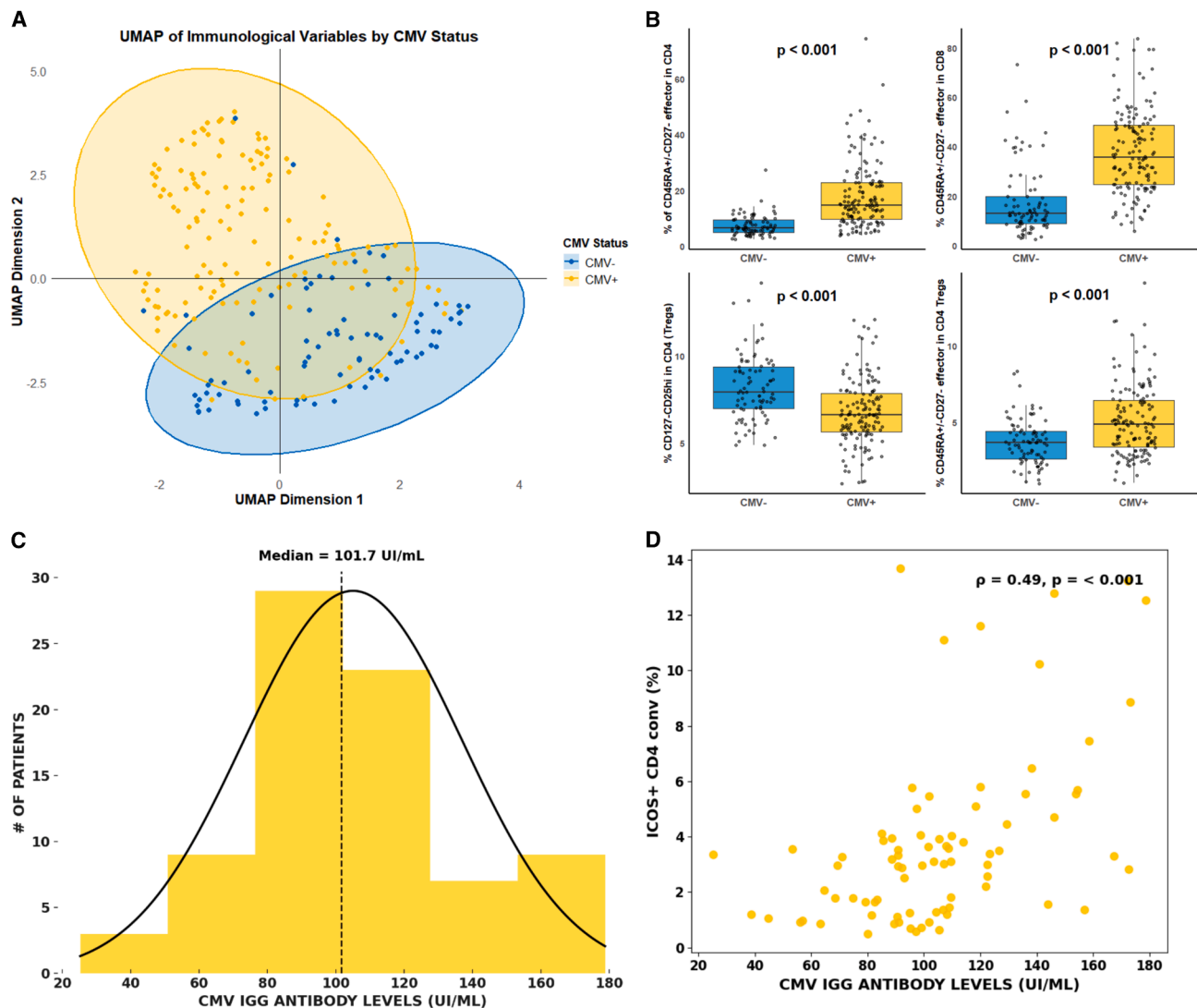


Figure 4. CMV seropositivity associates with a lower amount of circulating regulatory T cells

uMAP of immunological variables by CMV status (A), boxplots of proportions of circulating immune populations significantly impacted by CMV (B), distribution of IgG levels according to the number of patients (C), and Spearman's correlation between the proportion of ICOS+ in CD4 conv T cells and CMV IgG antibody levels (D).

association, as illustrated by the absence of association in patients with melanoma treated with both anti-CTLA4 and anti-PD1. The treatment of TNBCs has evolved significantly in the past decade, and now standards include anti-checkpoint inhibitors (ICIs) in addition to NAC. Given the better prognosis observed in patients with CMV seropositive metastatic melanoma treated by anti-PD1 blockers,²¹ it will be crucial to determine whether this is also the case in patients with TNBC treated by NAC and immune checkpoint blockers.

Limitations of the study

This study has several limitations. First, although the design was prospective, it was conducted across only two centers, which may limit the generalizability of the findings. Second, although most patients in the recent cohort received neoadjuvant chemo-

therapy, a subset was treated with upfront surgery. This treatment approach was consistent with the standard of care at the time of inclusion, but it does not fully reflect current clinical practice, which now recommends neoadjuvant chemo-immunotherapy for high-risk TNBC. Recent clinical guidelines have incorporated the use of immune checkpoint inhibitors (ICIs) in combination with chemotherapy as part of the neoadjuvant regimen, particularly following the KEYNOTE-522 protocol.^{4,6,30} As a result, the prognostic impact of CMV serostatus in patients treated with combined chemo-immunotherapy remains to be determined. We plan to evaluate this question in a future cohort, once sufficient follow-up data on recurrence events become available.

Together, our results highlight the impact of the history of infection with cytomegalovirus on TNBC relapses and overall

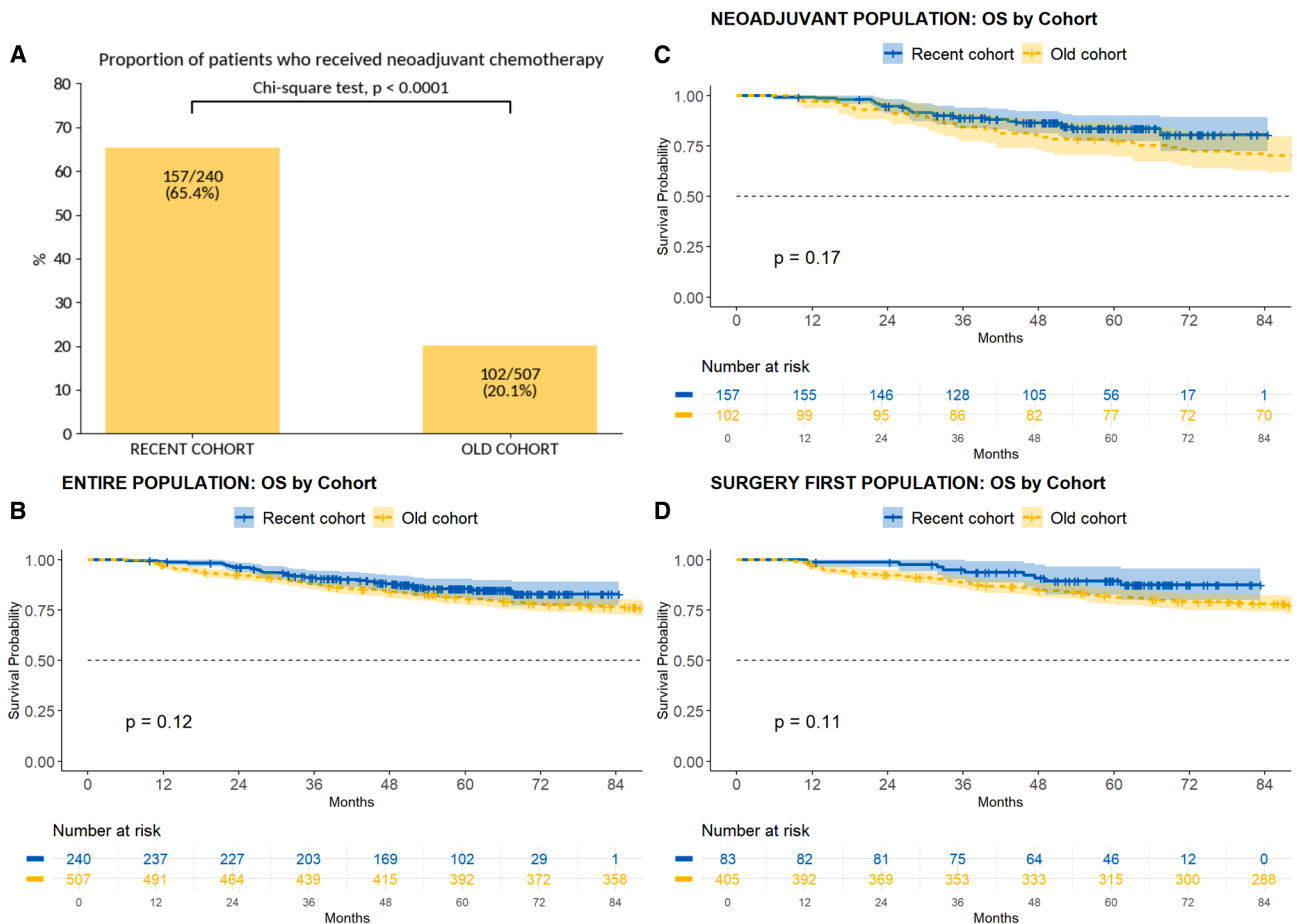


Figure 5. Therapeutic modalities and overall survival in the old and recent TNBC cohorts

Proportion of NAC in the two cohorts (A), Overall survival of the whole two cohorts (B), or split by therapeutic modalities, NAC (C), or primary surgery (D).

clinical outcome, establish an innovative prognosis score for stratifying patients, and identify decreased amounts of circulating regulatory T cells as a potential mechanism for the observed negative impact of CMV on the health of patients with TNBC.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Olivier Lantz (olivantz@curie.fr).

Materials availability

No specific materials were generated for this study. Further information and requests for resources and reagents should be directed to and will be fulfilled by the [lead contact](#).

Data and code availability

- All data reported in this article will be shared by the [lead contact](#) upon request in a manner preserving the anonymity of the patients.
- This article does not report original code.
- Other items will be shared by the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

R.D.S. performed statistical and computational analysis and prepared tables and figures. C.A. analyzed the immunological data and coordinated the analysis. O.L. coordinated and funded the immunomonitoring. H.R., I.D.D.S., D.G., D.L., and A.I.L. performed the immunomonitoring. M.M. provided the demographic and clinical data for the old cohort. L.C. and F.C.B. provided clinical

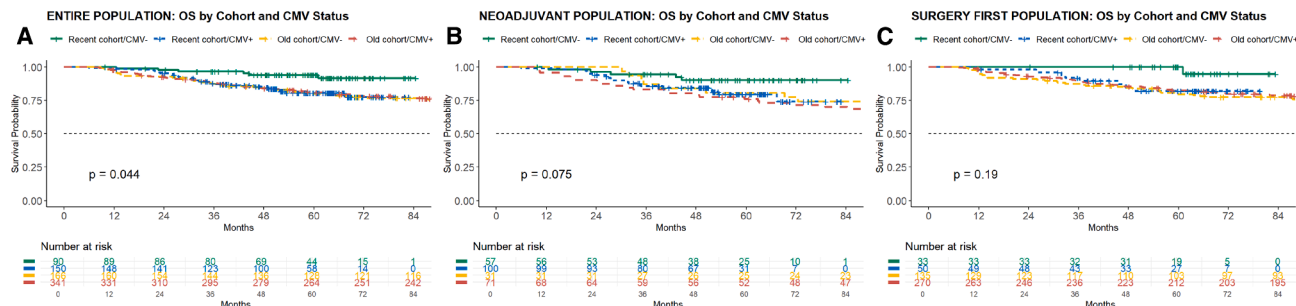


Figure 6. CMV seropositivity and overall survival in the old and recent TNBC cohorts
Overall survival of the whole two cohorts according to CMV status (A) or split by therapeutic modalities, NAC (B) or primary surgery (C).

interpretation of the results. I.B. provided critical human resources. F.R., E.L., C.B., N.P., M.P.S., J.Y.P., C.D., F.B., L.D., A.V.S., and C.L. participated in the construction of the cohort, including the collection of clinical information. C.L.T. built, funded, and supervised the organization of the recent cohort. C.A., R.D.S., and O.L. wrote the article. All authors read and approved the final version of the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TCRgdFITC	Becton Dickinson	Cat# 347903
TCRVa7.2 PerCP 5.5	Biologend	CAT# 351710
ICOS APC	Invitrogen	CAT# 17-9948-42
CD3 Alexa 700	Biologend	CAT# 300424
CD57APC Vio770	Miltenyi	CAT# 130-116-503
CCR7BV421	Biologend	CAT# 353208
TCR Va24 BV510	Biologend	CAT# 342918
CD27 BV605	Biologend	CAT# 302830
CD127 BV650	Biologend	CAT# 351326
PD1 BV711	Biologend	CAT# 329928
CD161BV785	Biologend	CAT# 339930
CD25 PE	Becton Dickinson	CAT# 555432
CD4 PE TX	Invitrogen	CAT# MHCD0417
CD8bPC5	Coulter	CAT# IM99512
CD45RAPC7	eBiosciences	CAT# 25-0458-42
Critical commercial assays		
CMV-IgG kit	DRG Instruments	EIA-3468
Software and algorithms		
RStudio	Posit Software	Version 2023.03.0
Other		
Clinical trial number	Clinical gov	NCT03017573

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Patients

Data for this study were prospectively collected as part of the SCANDARE trial (ClinicalTrials.gov ID: NCT03017573), which included patients (N=245) with biopsy-proven invasive triple-negative breast cancer (TNBC) of no-specific type who were referred at Institut Curie Hospital, France, from January 2017 to March 2021 (Figure 1). SCANDARE was approved by a national ethics committee (CPP Ile-de-France 3) (n°3440) and the French National Agency for the Safety of Medicines and Health Products (ANSM) in June 24th, 2016 (ID-RCB 2016-A01095-46). SCANDARE is a multiple cohort basket trial without therapeutic intervention. By participating in SCANDARE, patients receive standard treatments and agree to get additional sampling done along with their disease evolution. For this particular TNBC sub-cohort that will be called thereafter “recent” cohort, patients’ inclusion started in April 2017 and closed in March 2021 with a planned 5-year follow-up. All patients received standard treatment according to the stage of the disease and usual procedures. All participants provided written informed consent prior to their inclusion in the trial. After inclusion, a blood draw was done to perform both mononuclear cell isolation and multiparametric flow cytometry while the plasma was isolated to do a CMV serology. For the main analysis, we excluded five patients, including four who withdrew their consent and one with unknown CMV status. For the secondary analysis, we additionally excluded twelve patients because their immunological data were not available. We also retrieved another less-well characterized cohort (n=507) that we called “old” cohort corresponding to patients treated at Institut Curie for TNBC between March, 1st, 2001 and December, 30, 2005. During this period, in France, the regulatory framework for non-interventional studies on biological and clinical data obtained during regular diagnosis or therapeutic procedures was a “no objection” status. A booklet informing the patients of this framework was provided to all patients treated at Institut Curie during this period. Serums were obtained from diagnosis blood samples and kept frozen at -20° until CMV serology analysis in 2025.

METHOD DETAILS

Treatment and post-treatment surveillance of the prospective “recent” cohort

Two main treatment sequences were defined according to the administration of neoadjuvant chemotherapy (NAC, n=157) or upfront surgery (n=83). In the first group, as per standard of care, treatment consisted of neoadjuvant chemotherapy (anthracycline and taxanes), followed by surgery (breast-conserving surgery or mastectomy, with axillary lymph node dissection or sentinel lymph node technique) and radiotherapy. In the second group, treatment consisted of upfront surgery (breast-conserving surgery or mastectomy, with axillary lymph node dissection or sentinel lymph node technique), followed by adjuvant chemotherapy when indicated, and radiotherapy. After treatment, patients were regularly evaluated by clinical examinations, annual mammography, and follow-up tumor marker tests. Follow-up examinations were performed every 3 months for the first 2 years, then every 6 months for the next 3 years, and annually thereafter. When recurrence was suspected, Computed Tomography (CT), Magnetic Resonance Imaging (MRI), or Positron Emission Tomography (PET) imaging was performed, and biopsy of the lesion was recommended.

Clinical data for the recent cohort

Clinical data consisted of the patient age, body mass index and menopausal status. T and N stages were determined according to the 9th edition of the American Joint Committee on Cancer (AJCC) Staging Manual.³¹ Pathological characteristics of the primary breast cancer were obtained from biopsy samples, including hormone receptor status (estrogen-ER and progesterone-PR), HER2 status (HER2-0 or HER2-low: IHC score of 1+ or 2+ with a negative FISH result), Ki-67 index, and histologic grade. The threshold for estrogen and progesterone receptor negativity was <10%, in line with European recommendations.^{32,33} Molecular alterations were obtained using genetic testing for germline mutations, including at least BRCA1 and BRCA2.

Outcomes

The primary endpoint was recurrence-free survival (RFS), defined as the time from primary treatment (NAC or surgery) to the first occurrence of disease relapse—whether local, regional, distant, or any combination thereof—death from any cause, or the date of censoring at the last time the patient was known to be alive. Secondary endpoint analyses were related to overall survival (OS) and pathological complete response (pCR). OS was defined as the time from primary treatment (NAC or surgery) to death from any cause or censoring at the time of last known alive status. pCR was defined by the absence of invasive cancer in the breast and axillary nodes, irrespective of the presence of residual ductal carcinoma *in situ* (ypT0/is ypN0), as recommended by the EMA and FDA guidelines. The duration of follow-up was calculated from the date of the initial treatment (date of NAC beginning or date of upfront surgery) to the date of the last clinical consultation.

Clinical data for the retrospective “old” cohort

Clinical data were retrieved from the Institut Curie information system. Patients initially treated for non-metastatic breast cancer for whom serum frozen at initial treatment was available were included in this retrospective study. Patients were also selected according to availability of a minimal set of clinical and outcome information including ER, RP and HER2 status as above, date of treatment, nodal status, primary or secondary surgery after NAC, date of surgery, survival status and date of death. This led to a n = 507 size cohort starting from a >850 serum collection. Most of the excluded cases corresponded to non-TNBC diagnosis or insufficient clinical information.

CMV serostatus

CMV serostatus was determined on plasma by enzyme-linked immunosorbent assay (ELISA) using a CMV IgG kit available at DRG Instruments GmbH (Germany) following manufacturer instructions (reference EIA-3468). The DRG CytoMegalovirus (CMV) IgG Enzyme Immunoassay Kit is an ELISA that includes negative and positive controls. Quantitative values are determined using 3 standards and a calibration curve. Positive values > 11 U/mL; negative values <9 U/mL; gray zone 9-11 U/mL.

Immunological data (only relevant for the recent cohort)

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep solution (StemCell), collected in PBS (Gibco) supplemented with 0.4g/L of human serum albumin and immediately processed for flow cytometry analyses. PBMCs were further stained with a 15-antibody panel including anti- TCRgd (BD), TCR Va7.2 (Biolegend), ICOS (Invitrogen), CD3 (Biolegend), CD57 (Miltenyi), CCR7 (Biolegend), TCR Va24 (Biolegend), CD27 (Biolegend), CD127 (Biolegend), PD1 (Biolegend), CD161 (Biolegend), CD25 (BD), CD4 (Invitrogen), CD8b (Coulter), and CD45RA (eBiosciences). Of note, in our panels we use antibody recognizing the CD8beta molecule and not the CD8alpha molecule in order to increase its specificity to T cells. Supervised analysis of 36 predefined subsets (see [Figure S1](#); [Table S1](#)) was performed using an automated in-house shiny software. Data were analyzed using Prism (GraphPad). UMAP on all 36 predefined subsets were performed on R using umap package (Posit Software, PBC. 2023, RStudio Version 2023.03.0).³⁴

QUANTIFICATION AND STATISTICAL ANALYSIS

In this exploratory observational study, no prespecified statistical plan was planned. All clinical and biological parameters were analyzed according to CMV serostatus. Biomarkers' values between patients with CMV+ or CMV- were compared using the Mann-Whitney test. To account for multiple testing ($n=36$), the Bonferroni correction was applied to control the false discovery rate. Median follow-up was estimated using the reverse Kaplan-Meier method.³⁵ Survival curves were estimated by the Kaplan-Meier method (log rank test). The prognostic impact of all factors measured at diagnosis was studied with Cox proportional hazards models for survival over time and linear dependency of the log-hazard function on covariates. Prognostic models for RFS and OS were built using backward variable selection. The likelihood ratio test (LRT) for added prognostic value of CMV status, T stage and N stage was obtained by comparing log likelihoods of multivariable prognostic models without and with CMV status (chi-square test: χ^2). OS was compared between the two cohorts (recent vs. old) using the Kaplan-Meier method, with differences assessed accordingly. Survival analyses were specifically conducted in patients treated with surgery first and in those who received neoadjuvant chemotherapy, with further stratification according to CMV status (CMV+ vs. CMV-). In addition, within the old cohort, the impact of CMV status and nodal involvement on OS was evaluated in these treatment-defined subgroups. All statistical tests were two-sided, with significance set at $p < 0.05$. Analyses were performed using RStudio (version 1.3.1073, 2009–2020 RStudio, PBC).³⁴