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Microbiology and Pathology

Infected Human Megakaryocytes

Pathways and Genetic Predispositions

Highlights {

Pathogenesis of Local Cold Injury

Regeneration and Growth of Planaria

Discovering Thoughts, Inventing Future

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Hepatitis C Virus Infected Human Megakaryocytes and Platelets: Intra-and Extracellular Evaluation

By Caroline Mitiká Watanabe, Nathália Almeida Souza Tancler, Aline Márcia Marques Braz, Shelly Favorito de Carvalho, Giovanni Faria Silva, Maria Inês de Moura Campos Pardini, Maria Aparecida Custodio Domingues, Guilherme Targino Valente, Paulo Eduardo de Abreu Machado, Rejane Maria Tommasini Grotto & Marjorie de Assis Golim

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Abstract- This study aimed to determine whether the hepatitis C virus (HCV) infects human megakaryocytes and platelets and to measure the expression of receptors involved in virus-cell interaction. Platelets from healthy donors were infected with HCV in vitro and analyzed for viral expression and the receptors claudin-1 and cluster of differentiation 81 (CD81). HCV was detected on the surface and cytoplasm of both cells; cytoplasmic expression was higher compared to the surface. Platelets presented a claudin-1+/CD81-phenotype, and megakaryocytes showed aclaudin-1+/CD81+ phenotype. We conclude that megakaryocytes and platelets are susceptible to HCV infection, regardless of CD81 expression, and megakaryocytes may serve as possible sites of viral replication.

Keywords: viral infection, megakaryocytes, platelets, hepatitis C virus, chronic hepatitis C.

GJMR-C Classification: NLM: WC 536

HEPATITISCVIRUSINFECTEDHUMANMEGAKARYOCYTESANDPLATELETSINTRAANDEXTRACELLULAREVALUATION

Strictly as per the compliance and regulations of:

Hepatitis C Virus Infected Human Megakaryocytes and Platelets: Intra-and Extracellular Evaluation

Caroline Mitiká Watanabe [α], Nathália Almeida Souza Tancler [σ], Aline Márcia Marques Braz [ρ], Shelly Favorito de Carvalho [ω], Giovanni Faria Silva [¥], Maria Inês de Moura Campos Pardini [§], Maria Aparecida Custodio Domingues [χ], Guilherme Targino Valente [ν], Paulo Eduardo de Abreu Machado [Θ], Rejane Maria Tommasini Grotto [ζ] & Marjorie de Assis Golim [£]

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Keywords: viral infection, megakaryocytes, platelets, hepatitis C virus, chronic hepatitis C.

I. Introduction

Despite significant advances in the treatment of chronic hepatitis C with direct-acting antiviral therapy [1,2], approximately 290,000 people died from disease complications in 2019, mainly from causes related to cirrhosis and hepatocellular carcinoma. Around 58 million people chronically carry the hepatitis C virus (HCV), and annually it is reported 1.5 million new cases. Therefore, hepatitis C is still a global public health problem [3].

HCV is hepatotropic and mainly targets hepatocyte cells. However, extrahepatic viral locations are reported in endothelial cells [4], gastrointestinal mucosal cells [5], fibroblasts [6], macrophages, dendritic cells [7-11], erythrocytes [12], peripheral blood mononuclear cells (especially B- and T-lymphocytes, monocytes [13]), platelets [13;14] and megakaryocytes [2]. The existence of extrahepatic reservoirs may help to understand the symptoms or extrahepatic manifestations that patients develop. Moreover, extrahepatic reservoirs may be related to the high rate of chronic infection [2,4,14-17]. Thrombocytopenia is a common extrahepatic manifestation among patients with chronic hepatitis C, particularly those with long-term complications. This condition increases the risk of bleeding and complicates liver biopsies, which leads to compromised patient follow-up [18,19]. Acquired thrombocytopenia is trigged by several causes, including diseases caused by infectious agents such as HCV, HIV, and Helicobacter pylori [20,21]. The pathophysiology of thrombocytopenia may involve factors such as increased platelet destruction, decreased platelet production, bone marrow suppression, hypersplenism (including platelet sequestration in the enlarged spleen secondary to portal hypertension), autoimmunogenicity, thrombopoietin production imbalance, and adverse therapeutic effects [18, 22].

HCV's presence in bone marrow [22] and platelets [23] suggests these areas as biological compartments for the virus. Thrombocytopenia in chronic hepatitis C patients is associated with HCV's infection of, or adhesion to, platelets [14,23]. This interaction impairs both platelet function and the production of megakaryocytes, which are essential for platelet formation [2,25]. Platelet recovery after viral suppression/eradication further indicates the involvement of virus-platelet interactions in thrombocytopenia [20]. Patients with chronic hepatitis C also present alterations in the bone marrow microenvironment. Patients with a high viral load present hypo- and hypercellularity associated with sedimentation of immune complexes and peripheral cytopenia [22]. However, the exact mechanism of the HCV interaction with platelets—whether the virus only adsorbs to the platelet membrane or is internalized there—is poorly understood. Additionally, the virus can infect megakaryocytes in the bone marrow, transferring to platelets during thrombocytogenesis. Evidence of HCV in bone marrow [22] and platelets [23], along with

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susceptibility to in vitro infection in the megakaryocytic lineage (MEG-01) [2] and platelets [14] supports this view.

Considering the lack of evidence regarding the intra- and extracellular HCV localization and the receptors involved in the virus-target cell interaction, we evaluated the presence of intracellular and extracellular HCV in megakaryocytes and platelets, and the expression of the receptors used by the virus, cluster of differentiation 81 (CD81) and claudin-1. We sought to clarify whether HCV is only adsorbed on the surface of megakaryocytes and platelets or whether these cells are susceptible to infection. Moreover, we explored the potential link between this viral interaction and the thrombocytopenia often observed in infected patients.

II. Material and Methods

a) Ethical aspects

The Research Ethics Committee of the Botucatu Medical School (UNESP) approved this study under protocol 1.354.285. All donors and patients participating in the study provided their consent by signing informed consent forms. All experimental assays were performed at the Laboratory of Applied Biotechnology, Clinical Hospital of the Botucatu Medical School, UNESP.

b) Megakaryocyte and platelet isolation

Megakaryocytes were sourced from six bone marrow donors at Amaral Carvalho Hospital in Jaú, Brazil. These cells were harvested from the donors' iliac crest concurrently with their bone marrow donation for transplantation. Bone marrow samples (4 mL) were collected with modified CPDA-1 anticoagulant (CPDA-1 with 6% EDTA) diluted 1:1 with megakaryocyte buffer (PBS solution with 1% D-glycoside, 3% sodium citrate and 13.5% bovine serum albumin at 22%) [26,27,28]. The diluted bone marrow was sterile filtered through a nylon filter (160μm) and carefully placed on Percoll® (Sigma-Aldrich) in equivalent proportions (1:1). The samples were then centrifuged at 405xg for 20 minutes at 20°C. Megakaryocyte pellets were collected from the top layer of Percoll® and immediately washed with the double volume of megakaryocytes buffer (4°C). All washed pellets were centrifuged at 405xg for 10 minutes at 4°C. The enriched megakaryocyte pellet was resuspended in Roswell Park Memorial Institute Medium (RPMI) 1640 with L-glutamine and 1% antibiotics (Gibco) until in vitro infection.

Peripheral blood platelets were collected with EDTA anticoagulant (BD Vacutainer®- 5mL) from healthy donors (n=4) and processed as described by Padovani et al.[14]. Briefly, fresh blood samples were centrifuged at 700xg for 3 minutes at room temperature to obtain platelet-rich plasma, followed by a second centrifugation at 1600xg for 5 minutes (room temperature) to obtain platelet pellet. Cells were washed four times with saline (0.9% NaCl) and resuspended in RPMI 1640

supplemented with L-glutamine and 1% antibiotics (Gibco).

c) In vitro infection of megakaryocytes and platelets

For in vitro megakaryocyte infection, $4x10^6$ nucleated cells of the enriched megakaryocyte pellet were resuspended in 1 ml of RPMI 1640 medium. Then 6mL of HCV plasma genotype 1 (100,000 viral RNA copies/mL) was added to the flask culture. Cells were incubated for 36 hours in a conventional cell incubator (Thermo Fisher) at 37°C in 5% CO_2. During the infection period, the cell culture was or bitally agitated (18 hours) without changing the culture medium. HCV-negative plasma was used as a control.

In vitro platelet infection was performed as described by Padovani et al. [14]. Briefly, 1 mL of resuspended platelets was incubated with 1 mL of genotype 1 HCV plasma containing 100,000 RNA copies/mL from patients with positive RT-PCR. Samples were incubated in a shaker (New Brunswick Scientific) for 48 hours at 37°C with continuous shaking at 10xg.

d) Assessment of HCV infectivity in megakaryocytes and platelets

The presence of HCV in megakaryocytes and platelets was examined following in vitro infection. This analysis was conducted using flow cytometry and confocal microscopy. The purpose of these assessments was to confirm the susceptibility of these cells to the virus and to determine the viral location. For HCV detection, we used the monoclonal antibody NS4A-FITC (clone S4-13 - Abcam), a protein common to all HCV genotypes [29]. Molecular biological assay was performed only in megakaryocytes, since previous study has shown HCV RNA expression in platelets, as described in Padovani et al. [14].

i. Flow cytometry analysis

Following infection, megakaryocyte cells were labeled with anti-human-CD61-PE (VIPL2 clone, EXBIO) and anti-CD45-PerCP (HI30 clone, BD Pharmingen) [26]. To label platelets, we used only the anti-human-CD61-PE (VIPL2 clone, EXBIO), a commonly used marker for both platelets and megakaryocyte cells. The presence of HCV was evaluated using the monoclonal anti-hepatitis C virus antibody NS4A-FITC (S4-13 clone - Abcam). Cells were incubated for 30 minutes at room temperature in the dark, according to the manufacturer's instructions. In this assay, the control group comprises megakaryocyte cells incubated with plasma without HCV. Peripheral platelets obtained from HCV patients (n=2) were used to compare the virus behavior in vitro and in vivo. To detect HCV, we first labeled the surface of megakaryocyte and platelet cells. Then, we fixed the cells with 4.2% paraformaldehyde for 30 minutes at room temperature. Both cell types were permeabilized with 0.4% Triton X-100 for 5 minutes at room temperature and then stained with anti-NS4A. During the

steps, we washed the cells with the respective buffers following the rotation described above. To prevent the formation of platelet clots, polystyrene tubes were coated with 22% bovine albumin [30]. We obtained both cell types using a FACSCalibur™ device (BD Bioscience) and analyzed the results using CellQuest™ and FlowJo™ software (BD Bioscience). 50,000 events were collected at the CD61+ gate for all samples. The isotype controls were conducted following the experimental protocol and included Mouse IgG1-FITC (clone MOPC-21), Mouse IgG1-PerCP (clone MOPC-21), and Mouse IgG1-APC (clone MOPC-21 - BD Pharmingen).

The intra- and extracellular expression of HCV antigen (NS4A) was determined by comparing the mean fluorescence intensity (MFI - absolute number) values using the flow cytometry crossmatch assay model[28]$\frac{MIF\ NS4A\ intracelular}{MIF\ NS4A\ surface}$. Expression index value >1.0 indicates intracellular expression due to the increase in fluorescence compared to surface expression.

ii. *Indirect immunofluorescence staining of HCV-infected cells (confocal microscopy)*

Twenty-five microliters of labeled megakaryocytes and platelets were applied to silanized HDA slides and covered with Fluoroshield histology mounting medium (F6182-Sigma-Aldrich). The slides were sealed with resin, stored at 6°C ±2°C, and protected from light. Paraffin-embedded liver fragments from HCV-positive patients' biopsies were used as a positive control in this experiment. First, paraffin-embedded liver tissues were sectioned at a thickness of 0.3μm and fixed onto silanized HDA slides. The slides were then incubated using an antigen retrieval PT Link device (Dako) for 60 minutes at 65°C in a histological incubator at the Immunohistochemistry Laboratory of the Department of Pathology of the Clinical Hospital of the Botucatu Medical School - UNESP. To block non-specific binding, avidin and biotin (Vector Laboratories) were applied to coverslip slides for 20 minutes at room temperature. The slides were washed with PBS and labeled for two hours at room temperature with anti-human CD61-PE diluted 1:50 with EnVisionTM FLEX Antibody Diluent (Dako). After labeling, slides were washed and incubated overnight with EnVisionTM diluted monoclonal anti-hepatitis C virus antibody NS4A-FITCdiluted 1:20. Then, the slides were covered with Fluoroshield Histology Mounting Medium (Sigma-Aldrich) and kept at 4°C until confocal analysis. Images were taken using the TCS SP5 - Leica Laser Scanning Confocal Microscope, using the LAS AF software version 2.7.3.9723, available at the Electronic Microscopy Center of the Botucatu Institute of Biosciences, UNESP.

iii. *Detection of HCV RNA in megakaryocytes using real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)*

Part of the megakaryocyte-enriched cell suspension was divided equally. Tube 1 (negative control) was incubated with 1 mL of HCV-negative plasma. Tube 2 (test condition) had megakaryocyte added to RPMI and incubated with a pool of plasma containing 100,000 IU/mL of HCV genotype 1. Tube 3 (negative control) had 1 mL of the 100,000 IU/mL HCV genotype 1 plasma pool. The purpose of the final control assay was to prevent the adsorption of RNA present in the plasma in the tube; therefore, no megakaryocyte cells were added. The tubes were washed five times to remove any free virus that did not aggregate in the cells. All supernatants from each step were collected and frozen at -80°C for subsequent quantification of HCV RNA by qRT-PCR using the Abbott RealTime HCV assay (Abbott Molecular). The genomic region of the HCV 5'UTR was analyzed by nested PCR reaction, with HCV RNA being converted to complementary DNA using the High-Capacity cDNA Archive kit (Applied Biosystems). All procedures were carried out according to the instructions provided by the manufacturer.

e) *Evaluation of the expression of key HCV entry receptors claudin-1 and CD81*

We evaluated the expression of Claudin-1 and CD81, key cellular entry receptors used by HCV to infect cells. Flow cytometry and confocal microscopy were used to examine megakaryocytes and platelets receptors. To perform immunophenotyping, monoclonal antibodies were used along with phenotypic markers, as previously described. Specifically, anti-human Claudin-1Alexa Fluor 488 (clone 2H10D10, RheaBiotech) and anti-human CD81-APC (clone M38, EXBIO) were used.

f) *Statistical analysis*

Descriptive statistical analysis, including means, standard deviations, and ranges (minimum and maximum), was performed with Prism 8 software (GraphPad®).

III. Results

a) *HCV-infected megakaryocytes and platelets*

Efficient HCV infection of megakaryocytes and platelets was demonstrated in vitro through the detection of viral expression (NS4A) and viral load (mean 34.25 IU/mL) [31]. The objective was to demonstrate the biological event through descriptive analysis. In megakaryocytes, the average percentage of NS4A in the membrane was 51.71% ± 26.72 (range 11.01%-85.96%). For the cytoplasm, this percentage was 75.57%±29.66 (range 26.61%-99.77%) (Figure 1-A). The NS4A MFI in the membrane was 30.23±9.03

(range 19.11-43.71), while in the cytoplasm, the MFI was 63.32±32.41 (range 21.29-107.5).

The expression of intracellular viral NS4A was 2.09 times higher than that of membrane NS4A (Figure 1-B), indicating a higher concentration of NS4A or HCV in the intracellular compartment. Supporting this observation, confocal microscopy images showed the presence of NS4A on the megakaryocyte membrane (Figure 1-C1), along with CD61 (a megakaryocyte characterization marker) (Figure 1-C2) and 7AAD (a nuclear marker) (Figure 1-C3). By examining the Z-axis (depth) of the cells, we observed a high brightness density of NS4A expression inside the cell (Figure 1-C4), corroborating the findings from the flow cytometer analysis.
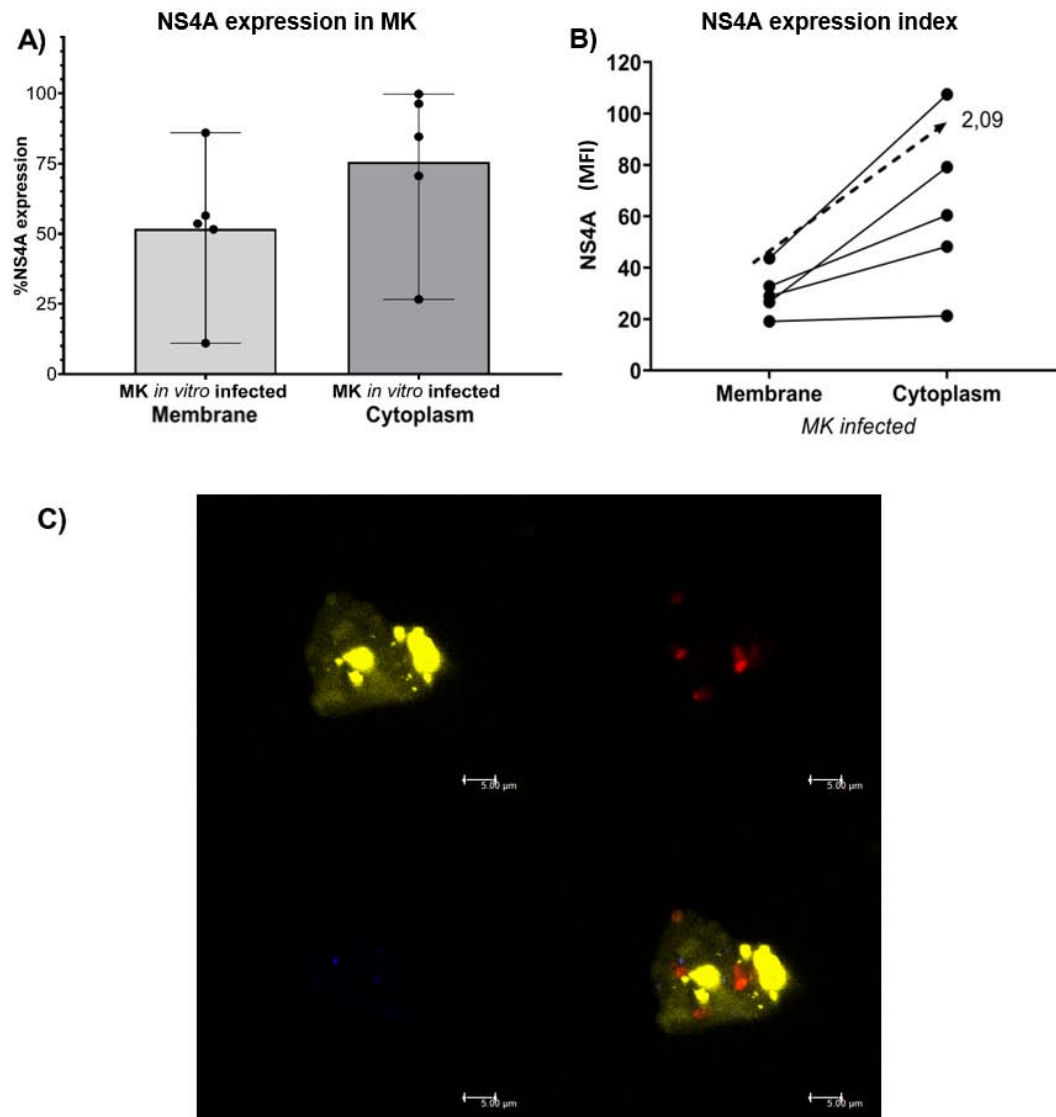
Figure 1: In vitro HCV-infected megakaryocytes were examined by flow cytometry and confocal microscopy. The percentage of NS4A expression was 51.71%±26.72 (range 11.01%-85.96%) in the membrane and 75.57%±29.66 (range 26.61%-99.77%) in the cytoplasm. In the membrane, the mean fluorescence intensity (MFI – absolute value) of NS4A was 30.23±9.03 (range 19.11-43.71). In the cytoplasm, the MFI was 63.32±32.41 (range, 21.29-107.5). The expression index NS4A in the cytoplasm was 2.09. Confocal images show the expression of NS4A in yellow, CD61 in red, 7AAD in blue, and overlay markers in the sample. MK: megakaryocyte; MFI: mean fluorescence intensity.

In vitro infected platelets expressed NS4A on both the membrane (47.89%±11.66, range 37.22%-63.85%) and cytoplasm (75.41%±8.36, range 68.73%-86.43%), similar to what was observed in megakaryocytes (Figure 2-A). The NS4A MFI was higher inside the cell (40.82±9.37; range 32.2-53.28) than in the membrane (18.32±2.89; range 15.12-21.29) (Figure 2-B). The expression index NS4A in the cytoplasm was 2.22. Confocal images of in vitro infected platelets showed co-expression of NS4A and CD61 on the surface and in the cytoplasm of the cells (Figure 2C).
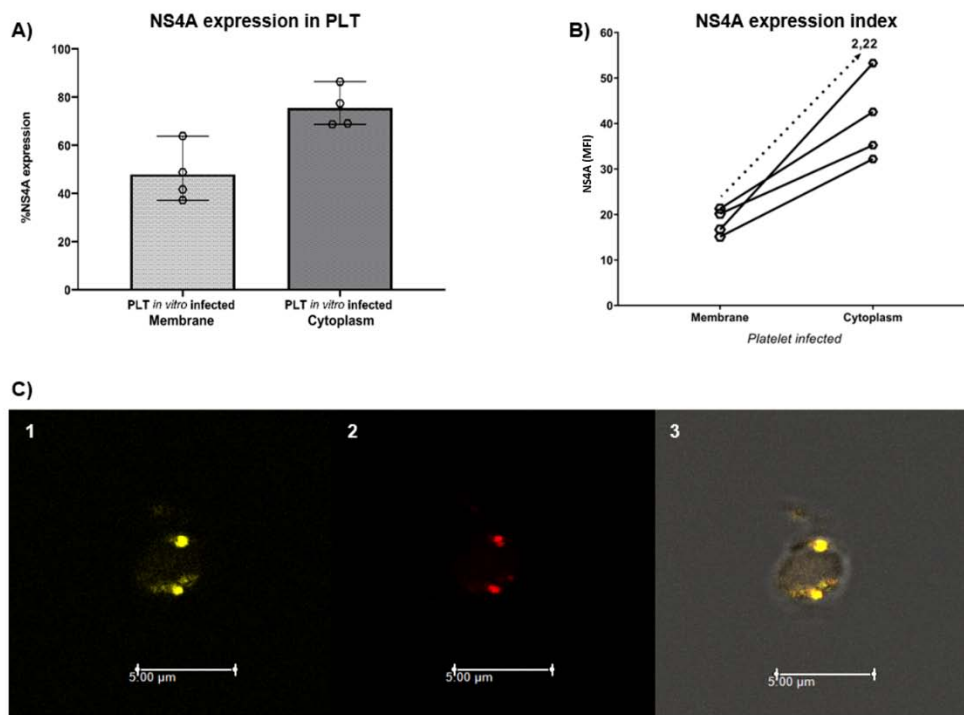
Figure 2: Platelets infected with HCV were evaluated in vitro using flow cytometry and confocal microscopy. The percentage expression of NS4A (A) was observed in the membrane (47.89%±11.66, range 37.22%-63.85%) and in the cytoplasm (75.41%±8.36, range 68.73%-86.43%). The median fluorescence intensity of NS4A (B) in the membrane (18.32±2.89, range 15.12-21.29) and in the cytoplasm (40.82±9.37, range 32.2-53.28) indicated an increase ratio of 2.22. The confocal images (C) obtained show the expression of NS4A in yellow (1), CD61 in red (2), and the overlay of both (3). PLT: platelet; MFI: mean fluorescence intensity.

To analyze viral expression in platelets in vivo, we evaluated samples from HCV-positive patients with detectable viral loads (n=2). Patient 1 had an HCV genotype 3 and a viral load of 17.051 IU/mL (4.23 log). Patient 2 had genotype 1B with a viral load of 3.243.950 UI/mL (6.51 log). According to the *in vitro* findings, platelets express higher viral concentrations in the cytoplasm compared to the cell surface. The results of the histological evaluation demonstrated the co-expression of CD61 and NS4A in liver tissue from HCV+ patients (Figure 3), reinforcing the idea that platelets can carry the virus and serve as extrahepatic reservoirs.
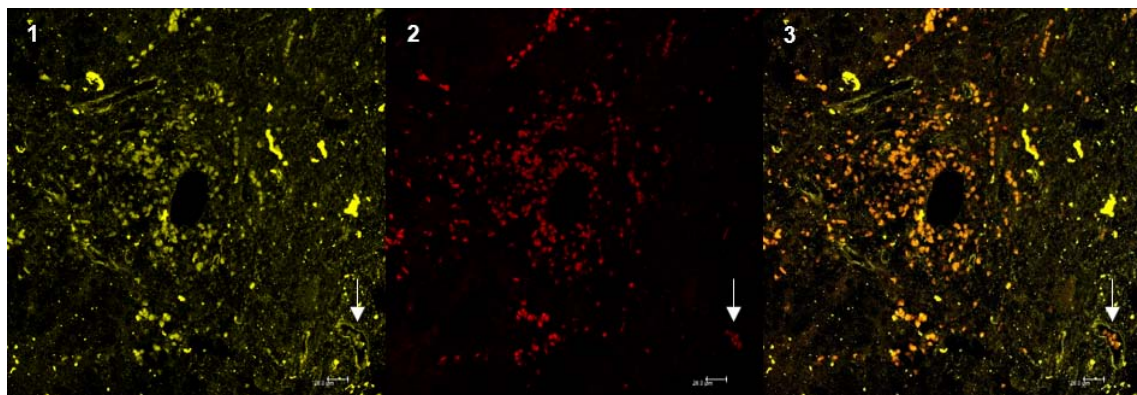
Figure 3: Viral expression in platelets in liver fragments from HCV patients: histological analysis using fluorescent markers. 1) Expression of NS4A on platelet surface; 2) CD61, platelet marker; 3) Overlay of NS4A and CD61. The arrows indicate HCV+ platelets in the blood vessels.

b) *Megakaryocytes express claudin-1 and CD81, whereas platelets express only claudin-1.*

The main entry receptors associated with HCV infection are claudin-1 and CD81 [32,33]. In this study, both HCV-infected and uninfected megakaryocytes showed expression of these receptors. Claudin-1 was expressed in 79.47%±10.42 of HCV-infected megakaryocytes (with an MFI of 27.97±5.97) and 69.75%±26.49 of uninfected megakaryocytes (MFI 21.24±7.04). CD81 was expressed in 61.10%±14.05 of HCV-infected megakaryocytes (MFI 220.89±193.95), and 67.56%±13.39 of uninfected megakaryocytes (MFI 105.47±86.46). It is important to emphasize that the in vitro infection process did not induce loss of expression of these receptors, as there was no significant difference between uninfected and infected megakaryocytes (Figure 4A, 4B, 4D). However, higher rates of CD81 (MFI) receptors in the presence of the virus suggest the involvement of immunomodulatory mechanisms.

Platelets expressed claudin-1 and were negative for CD81, confirming the expected phenotype. For in vitro infected platelets, the mean claudin-1 expression was 47.16%±10.36 and MFI was 17.65±4.37, whereas claudin-1 expression in uninfected platelets was 44.83%±8.26 and MFI was 16.36±1.27 (Figure 4C).

CD81, also known as TAPA-1, has four transmembrane domains that play a role in structural adhesion, activation, proliferation, and cell differentiation. According to microscopy images, the membranes of megakaryocytes expressed CD81, highlighting some cell regions with clusters forming structures known as tetraspanin-enriched microdomains. These microdomains are highly dynamic areas on the cell surface that facilitate interaction with other membrane components, crucial for the entry of HCV (Figure 4D-3) [33-34].

Unlike megakaryocytes, platelets expressed claudin-1 and are negative for CD81. For platelets infected in vitro with HCV, claudin-1 expression was 47.16%±10.36% (range 37.08%-57.89%) and MFI was 17.65 ± 4.37 (range 13.7-23.5). Uninfected platelets (exposed to HCV-negative plasma) showed a claudin-1 expression of 44.83%±8.26% (range 33.07%-52.42) and an MFI of 16.36 ± 1.27 (range 14.86-17.94) (Figure 4C). There was no significant difference in claudin-1 expression between HCV-infected and uninfected platelets. Confocal analysis showed a homogeneous distribution on the platelet surface (data not shown).
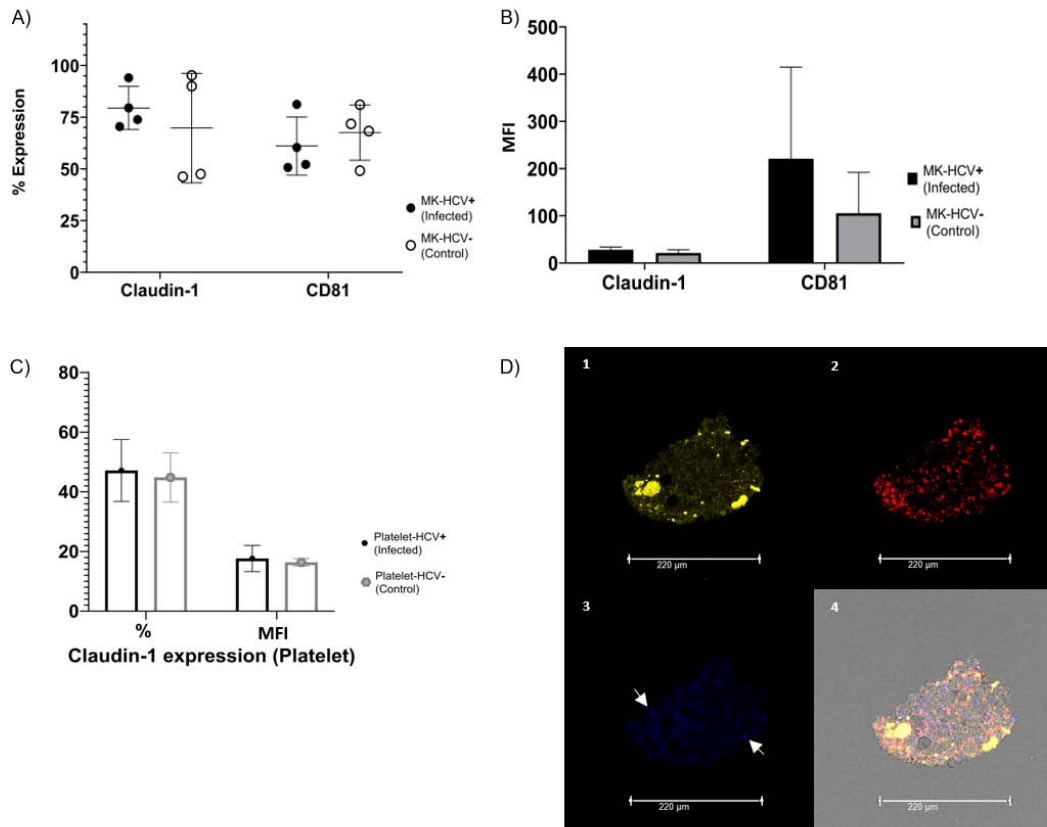
Figure 4: Expression of Claudin-1 and CD81 in megakaryocytes and platelets. (A) Percentage expression of claudin-1 (left) in infected megakaryocytes 79.47%±10.42% (range 70.46%-94.07%) and uninfected megakaryocytes (control) 69.75%±26.49% (range 46.26%-95.24%) and CD81 (right) in infected megakaryocytes 61.10%±14.05%

(range 52.15%-81.01%) and uninfected megakaryocytes (control) 67.56%±13.39% (range 49.15%-81.01%); (B) MFI Claudin-1 expression in infected megakaryocytes 27.97±5.97 (range 21.0-33.38), uninfected megakaryocytes 21.24±7.04 (range 14.99-28.39) and MFI CD81 220.89±193.95 (range 41.42-403.15) and uninfected megakaryocytes (control) 105.47±86.46 (range 36.85±231.86) (C) Claudin-1 expression on platelets; (D) Confocal images of megakaryocytes: 1- Claudin-1 (yellow); 2- CD61 (red); 3- CD81 (blue) and tetraspanin microdomain (white arrows); 4- overlapping markers. MK: megakaryocyte; MFI: mean fluorescence intensity; HCV: hepatitis C virus.

IV. Discussion

HCV was detected in both the surface and the interior of megakaryocytes, with clear areas of higher virus concentration. These findings confirm that megakaryocytes are susceptible to HCV. They expressed high levels of CD81, the major viral entry receptor. Furthermore, molecular analyses have shown that megakaryocytes can support viral replication by detecting the complementary strand of viral RNA. This corroborates previous studies [2,22,35,36] and characterizes the virus-permissive cell function.

Our results regarding viral expression on the surface and cytoplasm of megakaryocytes strengthen our hypothesis that HCV can infect platelet precursors. This suggests that the virus may be transmitted during the differentiation of megakaryocytes into proplatelets, generating infected young platelets, such as swine flu [37]. Our results align with those of Li et al., who studied thrombocytopenia in patients with hepatitis C [2]. They demonstrated that megakaryocytes from a patient with megakaryoblastic leukemia in vitro infected with HCV were permissive to the virus [2]. Moreover, electron microscopy analyses revealed that the virus tends to accumulate around the Golgi complex and vesicles present in megakaryocytes [2]. Our observations using confocal microscopy, which identified areas of higher virus concentration in megakaryoblastic, corroborate these findings.

Several cell lineages, including hepatocytes and megakaryocytes, present CD81 receptors. These molecules are distinguished from other transmembrane proteins by the presence of conserved regions in their extracellular domains. These regions enable CD81 receptors to associate with other proteins such as integrins, signaling molecules, and homo- and heterodimer proteins to form tetraspanin-enriched microdomains [38,39,40]. These microdomainsare coordinator molecules required for molecular trafficking, cell-cell fusion, motility, and signaling. Pathogens such as HCV and HIV use them as a gateway into the cell, facilitating viral entry [38-34].

The presence of HCV in the bone marrow correlates with the level of circulating viremia. Bone marrow changes such as hypo- and hypercellularity of erythroid, lymphoid, and myeloid lineages are attributed to factors such as viral load, viral subtype, immune status or immune complex deposition [19,22,36]. Abou El Azm et al. reported a decrease in the megakaryocyte population, the appearance of micromegakaryocytes,

and abnormalities in the proliferation and differentiation of hematopoietic stem cells [41]. These observations suggest that HCV can interact directly with these cell populations and contribute to peripheral thrombocytopenia [22]. Similarly, El-Barbary et al. (2010) found a decrease in megakaryocytic colony-forming units and thrombocytopenia in HCV+ patients [42]. Since we have shown that megakaryocytes are permissive for HCV infection, we can infer that the bone marrow may be the extrahepatic site of viral replication.

HCV infection impairs the functions of hepatocytes, the major producers and secretors of thrombopoietin, a growth factor involved in platelet control and formation. Thrombopoietin reduction directly affects megakaryocyte ploidy and development, reducing platelet production. The presence of viral RNA in megakaryoblasts and megakaryocytes further promotes thrombocytopenia. This can occur through cell death or via reduction of the thrombopoietin receptor c-Mpl (myeloproliferative leukemia protein) [2,43,44]. The interaction between megakaryocytes and HCV also facilitates the imbalance in circulating thrombopoietin levels due to the lack of consumer cells for this factor. Consequently, serum thrombopoietin levels are significantly higher in patients with chronic hepatitis C [42].

The presence of HCV in platelets was confirmed here and in previous in vitro and in vivo studies [14,24]. Furthermore, when analyzing the viral RNA in plasma and platelets of HCV-positive patients over 144 hours, there was a decrease in plasma viral concentration, while RNA levels in platelets remained the same [23]. This suggests that HCV can persist in the body for long periods when associated with platelets, which can be considered a viral reservoir. Here, we showed for the first time that platelets express HCV both on the surface and inside the cell. Since the virus does not express CD81, the major receptor involved in HCV infection [32,33,34,38,45,46], the primary question is how the virus infects the platelets.

The mechanism of HCV entry or interaction in platelets is still unknown. Studies have found that cells expressing CD81 (t-Molt4 cells) and those lacking this marker (pro-monocytic lineage U937 and platelets) exhibit similar binding rates to HCV [24]. This indicates that HCV can interact with cells in the absence of the CD81 receptor. HCV may interact with platelets through adsorption or association with adhesion molecules such as anti-human platelet antigen (HPA)-1 integrins [14,47]. This interaction could occur through platelet antigens

such as HPA-5b and HPA-1B, as their abundance is altered in platelets from hepatitis C patients [14,48]. Another interaction pathway may be through the glycoprotein VI receptor [37]. This receptor is a type 1 membrane glycoprotein belonging to the immunoglobulin (Ig) family with two Ig-C2 domains expressed on the membrane surface of platelets and megakaryocytes. IgG molecules associated with HCV could bind to the Ig-C2 domain, promoting not only cell-cell interaction but also viral dissemination and persistence [14,37,49].

Caludin-1, a junctional protein located on the apical and basolateral surfaces of hepatocytes, is an important receptor for HCV entry into these cells. Therefore, we investigated whether platelets and megakaryocytes express this protein. Claudin-1functions either directly by binding to CD81 or through cell-to-cell infection. Due to its cellular location, claudin-1 may be able to interact with HCV [50,51]. Our data showed that megakaryocyte cells express claudin-1and CD81, whereas platelets express only claudin-1. Cells expressingclaudin-1but lacking CD81 are susceptible to HCV [32]. Blocking the HCV-claudin-1 interaction with monoclonal antibodies prevented viral infection in vivo and in vitro, further implicating claudin-1in HCV infection [33].

Our findings indicate that claudin-1 expression in megakaryocytes is not uniform but rather concentrated in specific areas of the cell. HCV enters hepatocytes through the association between CD81 and claudin-1. This complex facilitates the formation of tetraspanin-enriched microdomains, which are concentrated in certain cell regions. Therefore, we inferred that a similar process of HCV entry might occur in megakaryocytes.

HCV infects hepatocytes either through cell-free particle diffusion followed by engagement with specific cellular receptors orvia cell-to-cell direct transmission mediated by mechanisms not well defined yet [52]. Cell-to-cell infection involves direct viral transfer to neighboring cells, allowing the virus to evade immune system cells and antibodies designed to fight it. This strategy may contribute to viral persistence [46,53-55].

CD81, claudin-1, occludin and low-density lipoprotein receptors are cellular molecules involved in cell-to-cell viral transmission. Since claudin-1was found to be expressed by platelets even in the absence of CD81, this pathway may be involved in platelet infection.[56]. Histological analysis of liver tissue from HCV patients revealed an accumulation of HCV-infected platelets (CD61+NS4A+) around blood vessels. The capillaries or vessels in the hepatic endothelium play a critical role in the exchange of macromolecules, solutes, and fluids from the blood and are also the entry point for pathogens into the liver. We believe that platelets can transport the virus across the endothelium to liver tissue, which could favor the interaction of the virus with different receptors, including CD81, claudin-1, and occludin, allowing viral internalization in hepatocytes [57].

V. Conclusion

This study demonstrated the presence of HCV on the platelet surface and, for the first time, identified viral expression in the cytoplasm of these cells. Possible mechanisms of interaction between HCV and platelets include adsorption due to platelet morphology/phenotype or interaction mediated by receptors such as claudin-1, even in the absence of CD81. Additionally, we demonstrated HCV on both the surface and inside of megakaryocytes, suggesting that platelets derived from infected megakaryocytes may enter the circulation with the virus.

Our findings contribute to understanding the pathophysiology of hepatitis C, especially the interaction of the virus with different cell types. The presence of HCV in megakaryocytes and platelets, either through direct viral interaction that induces apoptosis or by altering the maturation of progenitor cells, may be related to thrombocytopenia, an extrahepatic manifestation often observed in patients with chronic hepatitis C.

Abbreviation list:

CD81 - Cluster of differentiation 81

CPDA - Cit Phosph Dextrose Adenine
EDTA – Ethylenediaminetetraacetic acid
HCV - Hepatitis C virus
Ig - Immunoglobulin
MFI - Mean fluorescence intensity
RPMI - Roswell Park Memorial Institute Medium

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Overview of the Main Signalling Pathways and Genetic Predispositions Involved in Breast Carcinogenesis

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Summary- Breast cancer remains the most deadly cancer in women worldwide. It is a highly heterogeneous disease group, both biologically and molecularly. Mammary carcinogenesis is a multi-stage, complex and progressive process, involving the accumulation of several genetic and epigenetic abnormalities in oncogenes and suppressor genes. These abnormalities lead to activation or inhibition of various molecules involved in cellular and molecular signaling pathways, thus altering stem cell proliferation, differentiation and cell death. Patients with certain constitutional genetic abnormalities are a sub-population at high risk of accumulating several molecular abnormalities at an early stage, and of developing more invasive breast cancers. Understanding the molecular pathogenesis of breast cancer is an essential step towards distinguishing molecular subtypes with different prognostic and therapeutic implications. This review provides a synthesis of the major molecular abnormalities found in breast cancer, focusing on molecules that are considered in the literature as prognostic or theranostic markers.

Keywords: breast carcinogenesis - signaling pathways - mechanism of action - genetic predisposition.

GJMR-C Classification: NLM: WP 870

OVERVIEWOFTHEMAINSIGNALLINGPATHWAYSANDGENETICPREDISPOSITIONSINVOLVEDINBREASTCARCINOGENESIS

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Overview of the Main Signalling Pathways and Genetic Predispositions Involved in Breast Carcinogenesis

Imane Eliahiai [α], Mohammed Eljiar [σ], Jinane K Harmoum [ρ] & Mariame Chraïbi [ω]

Summary- Breast cancer remains the most deadly cancer in women worldwide. It is a highly heterogeneous disease group, both biologically and molecularly. Mammary carcinogenesis is a multi-stage, complex and progressive process, involving the accumulation of several genetic and epigenetic abnormalities in oncogenes and suppressor genes. These abnormalities lead to activation or inhibition of various molecules involved in cellular and molecular signaling pathways, thus altering stem cell proliferation, differentiation and cell death. Patients with certain constitutional genetic abnormalities are a sub-population at high risk of accumulating several molecular abnormalities at an early stage, and of developing more invasive breast cancers. Understanding the molecular pathogenesis of breast cancer is an essential step towards distinguishing molecular subtypes with different prognostic and therapeutic implications. This review provides a synthesis of the major molecular abnormalities found in breast cancer, focusing on molecules that are considered in the literature as prognostic or theranostic markers.

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I. Introduction

Breast cancer is the leading cause of cancer-related death in women. Over 80% of cases occur sporadically, underlining the importance of somatic abnormalities, which involve several risk factors related to the patient's lifestyle. Only 15-20% of breast cancers occur in a context suggestive of hereditary transmission of a mutation in a gene predisposing to the development of cancer. Mutation of the BRCA1 or BRCA2 genes is found in only 25% of cases. Thanks to extraordinary advances in molecular biology over the last few decades, our understanding of the cellular and molecular basis of cancer has broadened considerably. In this summary, the authors present a review of the signaling pathways most frequently involved in the carcinogenesis of sporadic forms of breast cancer, as well as the main predisposition genes found in hereditary forms.

II. Cells of Origin of Breast Cancer

The determination of the origin of breast cancer cells has been elucidated in the light of the understanding of the normal cell hierarchy. Mammary stem cells (MSCs), constituting a very small proportion of mammary gland cells, are undifferentiated and can produce new MSCs by self-renewal and give rise to a variety of differentiated cells by symmetrical and asymmetrical divisions. Asymmetric divisions give rise to ductular, alveolar and myoepithelial progenitor cells. Several processes of division with differentiation are initiated to give rise to mature ductular, alveolar and myoepithelial cells. In the healthy body, MSCs are involved in responding to cellular needs during reproductive life. This is thanks to a close interaction with their specific cellular microenvironment, known as the mammary stem cell niche [1].

Comparison of the established molecular characteristics of normal breast epithelial subpopulations with those of different breast cancer subtypes (luminal A, luminal B, HER2-positive, claudin-low, and basal-like), has provided an important framework for understanding the cellular origins of this cancer, both sporadic and hereditary. Cancer subtypes appear to aggregate along the hierarchy of normal cellular differentiation, starting with claudin-low undifferentiated tumors, followed by basal-like tumors, HER2 tumors and finally luminal A and B tumor subtypes [2]. On a molecular level, MSCs are similar to the low-claudin cancer subtype. The luminal progenitor subset has a molecular profile very similar to that of the tumor cells found in the basal-like subtype. The HER2, luminal A and luminal B subtypes reflect different cell types within the luminal lineage. The molecular profile of luminal A tumors is closest to that of mature luminal cells [2].

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With regard to familial breast cancer, it was initially proposed that the mammary stem cell resident in the basal layer of the mammary epithelium was the "cell of origin" of BRCA1-mutated basal-like tumors. This proposal was mainly based on histological studies that noted similarities between basal-like tumors and basal epithelial cells, i.e. the expression of basal cytokeratins and the absence of hormone receptor expression. Early work suggested that cells carrying the BRCA1 gene mutation show disturbed differentiation. Currently, analysis of the pre-neoplastic tissue of BRCA1 mutation carriers has revealed a target population predisposed to neoplastic transformation: an enlarged population of aberrant luminal progenitor cells. These cells show the greatest molecular similarity to normal luminal progenitor cells [2]. An experiment was carried out in 2011 involving transduction of human mammary epithelial cells with a cocktail of potent oncogenic lentiviruses, followed by implantation in humanized mammary adipose tissue. Results showed that BRCA1-mutated luminal cells were more susceptible to malignant transformation than basal cells, and produced predominantly basal-like tumors [3]. Luminal progenitor cells are reprogrammed to acquire basal-like characteristics. This is mediated in part by the BRCA1-regulated SLUG transcription factor. This epithelial-mesenchymal transition factor is overexpressed in tissues with a BRCA1 mutation and thus blocks luminal cell differentiation, directing the cells towards a basal fate [3].

Cell fate decisions along the mammary epithelial hierarchy may not be strictly unidirectional. Increasing evidence suggests that the dedifferentiation process can occur under non-physiological conditions. Luminal epithelial cells can convert to basal-like cells upon oncogenic stress in vivo, and induction of a P53 protein mutation in luminal cells produces tumors with basal-like features (Figure1). These data reflect the inherent plasticity of the mammary luminal compartment during carcinogenesis [4].
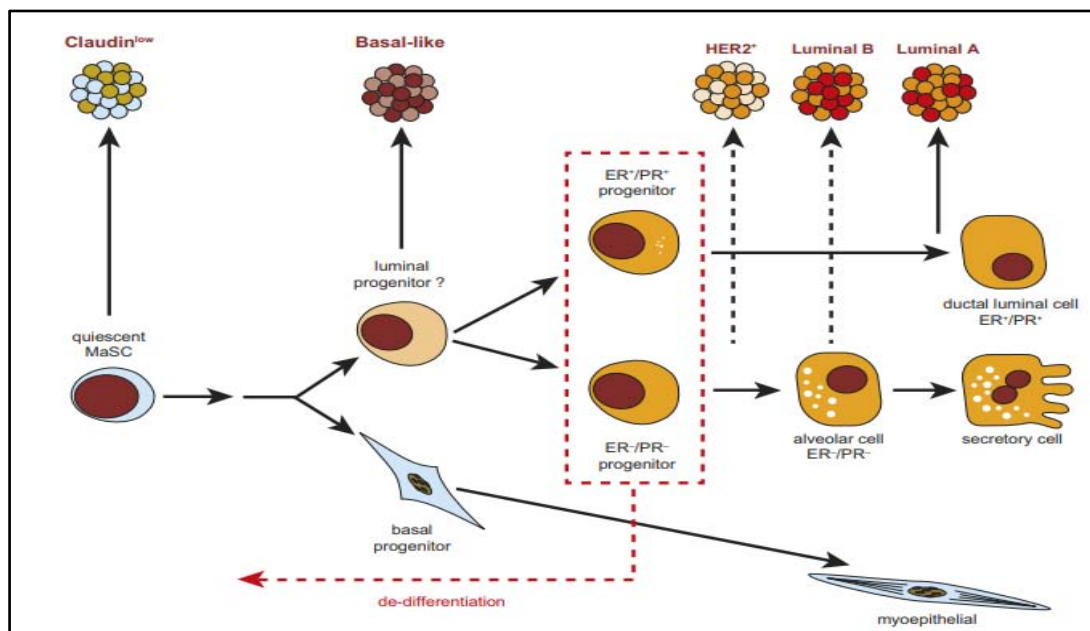
Figure 1: Schematic model of the relationships between cells of the breast epithelial hierarchy and breast tumour subtypes. Luminal progenitors also show marked de differentiation during oncogenesis. [2].

III. Signaling Pathways Involved in Breast Cancer Development and Progression

Cancer is caused by genetic and epigenetic alterations that disrupt cell signaling pathways. In this way, the tumor cell manages to escape the control mechanisms of proliferation, survival and migration. The main alterations in signaling pathways found in breast cancer stem cells are as follows:

A/Signaling pathways involving estrogen receptors

The estrogen receptor signalling pathway is the most common pathway in breast cancer. It involves estrogen ligands, which are transcription factors that activate or repress the expression of target genes upon receptor binding. There are two types of estrogen receptor: G protein-coupled membrane receptors and nuclear ERα, ERβ receptors. Normal breast tissue frequently expresses ERβ-type nuclear receptors. They are most often active as dimers. Although located on two different loci, ERα encoded by ESR1 located on the long arm of chromosome 6 and ERβ encoded by ESR2 located on the long arm of chromosome 14, these two receptors share common structural features.

In fact, ERα and ERβ are organized into 5 functional domains, designated A to F from the N-terminus to the C-terminus (Figure 2):

– The C domain includes the DNA Binding Domain (DBD), which binds to chromatin response elements known as EREs (Estrogen Responsive Elements). EREs are DNA sequences located at the promoter of target genes.

– The D domain, also known as the Hinge domain, ensures the receptor's flexibility at DNA level. It also contains a nuclear localization signal.

– The larger E domain contains the ligand-binding domain (LBD). It also ensures ligand-dependent transactivation of transcription (Activation Function 2 AF-2).

– The F domain, located at the C-terminus of the receptor, is still largely unknown. However, it could modulate ERα transcriptional activity and protein-protein interactions, notably with SRC-1 (Steroid Receptor Coactivator-1).
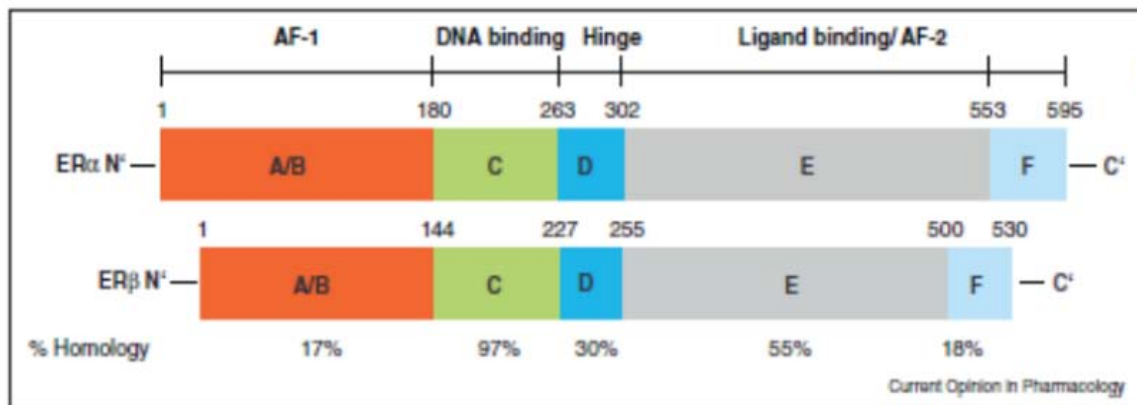
Figure 2: The functional domains of oestrogen receptors, numbered A to F (Pr Jacqueline LEHMANN CHE).

ERα and ERβ share 98% homology in their DNA-binding domains (DBD) (Pace et al., 1997), while they differ widely in their transcription activation domains (less than 15% homology in their N-terminal domains). Indeed, the N-terminal part of ERβ is about 40 amino acids shorter than that of ERα. Its AF-1 transcriptional activity is thus considerably reduced. As a result, ERα and Erβ recruit co-activator proteins differently, modifying their specific transcriptional effects.

ERα and ERβ share a 55-59% homology in their LBD (ligand-binding domain), which influences their affinity for their ligands. Moreover, ERα is associated with cell proliferation, whereas ERβ is thought to play an antiproliferative role [5].

1/ The genomic pathway

• Direct genomic pathway

The direct genomic pathway is activated when estrogen (E2) binds to its receptor, resulting in a conformational change. The conformational change of estrogen receptors facilitates the association and dissociation of enzymatic co-regulators. These proteins are either histone acetyltransferases or histone methyltransferases, or ATPase complexes such as SWI/SNF, which participate in chromatin remodeling. The receptor is then translocated into the nucleus and binds to specific DNA sequences, the EREs (Estrogen Responsive Elements), and activates the transcription of target genes via its AF-1 and AF-2 domains. A transcriptional machinery containing RNA polymerase II and TBP (TATA binding protein) is recruited to initiate transcription.

This stage requires the intervention of pioneering factors known as co-activators, including the main ones SRC, GATA3 and the FOXA1 protein. These proteins, especially FOXA1, play an important role in ERα binding to chromatin and activate transcription of genes involved in cell cycle progression, notably CCND1, which codes for cyclin D1 [6]. The latter is an important activator of cyclin-dependent kinases (CDK) 4 and 6, which coordinate cell cycle transition from G1 to S phase in many cancer cells (Figure 3). In breast tumor lines, it has been shown that ERα binds to chromatin even in the absence of estrogen, but in a FOXA1-dependent manner [6] The feedback loop between ERα and cyclin D1 may explain the mechanism of resistance to antiestrogen therapy, and justifies the use of kinase 4 and 6 inhibitors in combination with hormone therapy. [1].
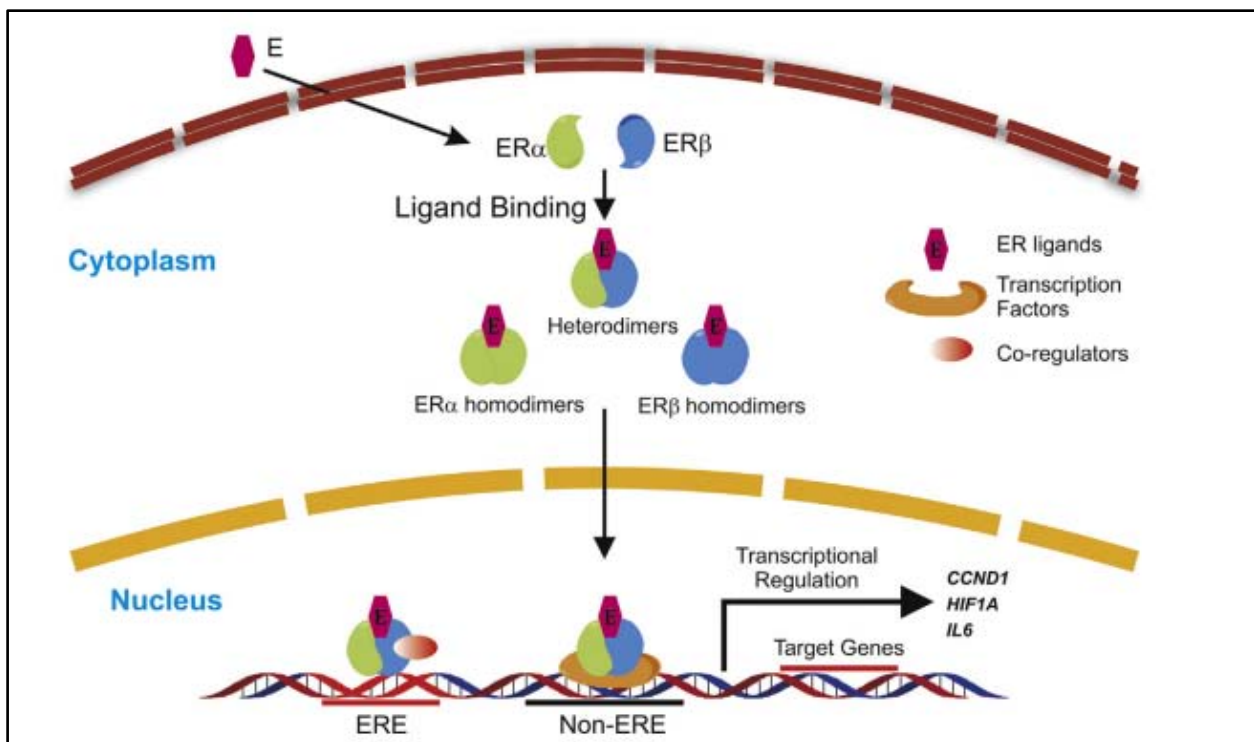
Figure 3: Direct genomic signalling pathway of oestrogen receptors ER: Nuclear hormone receptors form homo- or heterodimers on ligand binding and move into the nucleus for transcriptional regulation. ER dimersbind to the ERE region of target genes and recruitco-regulators such as FOXA 1 [1].

There are several isoforms with paradoxical effects in the regulation of ERα signaling. ERa36, the isoform most frequently found in metastatic breast cancer, is not blocked by tamoxifen. On the contrary, once bound to ERα 36, it promotes disease progression. The ERα 36 isoform accounts for 70% of resistance to hormone therapy [7].

- The indirect genomic pathway

A proportion of estrogen receptors activate the transcription of target genes by binding to other transcription factors already present on chromatin, without binding to ERE (Estrogen Responsive Elements) sites. This pathway is also known as the ERE-independent genomic pathway. In this case, the estrogen receptor acts as a transcriptional cofactor. The indirect genomic pathway is reserved for Erα, ERb have no coactivating activity. Several genes are activated by estrogen (E2) via the interaction of the estrogen nuclear receptor with transcription factors such as SP-1 (Specificity Protein 1), NF-κB (Nuclear Factor- κB), GATA1, STAT5 (Signal Transducer and Activator of Transcription 5) and AP-1 (Activating Protein 1). Genes activated in this way include those encoding IGF-1 (Insulin Growth Factor 1), cyclin D1, C-Myc protein, Bcl2 [8].

The activity of nuclear estrogen receptors can be modulated by signals other than estrogen. This is made possible by the ligand-independent AF-1 transactivation domain. Phosphorylation of estrogen receptors on serine 118 residues enables ERα to be localized at several target gene promoters to activate their transcription. This phosphorylation is induced either by type A and C protein kinases (PKA, PKC), cell cycle regulators, neurotransmitters, or growth factors such as EGF (Epidermal Growth Factor), IGF-1 (Insulin-like Growth Factor), TGFβ (Transforming Growth Factor) [9].

2/ the non-genomic route

The idea of the existence of a non-genomic estrogen-mediated pathway has been suggested since 1977, given that some estrogen-induced changes are too rapid to be mediated by the genomic pathway. A subset of ERα, localized to the plasma membrane, is involved in extranuclear signaling cascades. One of the most well-documented interactions is the ERα/Src interaction, which occurs rapidly after estrogen stimulation, leading to Src activation. Src is a tyrosine kinase which in turn phosphorylates RAS. This activation cascade induces the activation of MEK, which directly phosphorylates ERK1/2. Phosphorylated ERK1/2 migrates to the nucleus to activate genes such as CCND1 [8].

In addition to all the above-mentioned pathways, it has been shown that in the presence of estrogen, ERα rapidly interacts with the regulatory subunit of PI3 kinase (PI3K), enabling cells to enter the S phase of the cell cycle, and activate Cyclin D1. In 2012 a team studied the formation of the ERα/PI3K/Src complex in a cohort of 175 breast tumors. It showed that

activation of this complex was correlated with a poor prognosis and a low relapse-free survival rate [10].

B/ HER2 signaling pathways

HER2 is a proto-oncogene corresponding to a transmembrane protein encoded by the ERBB2 gene, located on the long arm of chromosome 17. HER2 belongs to the epidermal growth factor receptor tyrosine kinase family, which comprises 4 subtypes (EGFR)/HER1, HER2, HER3 and HER4. It controls cell growth, survival, differentiation and migration [11].

The molecular structure of the EGFR family consists of a large extracellular region, a single-span transmembrane (TM) domain, an intracellular juxtamembrane (JM) region, a tyrosine kinase domain and a C-terminal regulatory region. HER3 is the only tyrosine kinase-deficient receptor, which is why it assumes no signal transduction. The ligand for HER2 has not yet been identified. HER2 undergoes ligand-independent heterodimerization with the other 3 members of the EGFR family. At high HER2 concentrations, HER2 may undergo homodimerization due to its constitutively active conformation [12].

The formation of homodimers and heterodimers brings the intracellular domains closer together, resulting in asymmetric interaction of the intracellular kinase domain between the amino-terminal lobe of one tyrosine kinase and the carboxy-terminal lobe of the other, and promoting autophosphorylation of the tyrosine kinase domains. Several signalling pathways are then activated, including PI3K/Akt, MAPK, PLC γ, ERK1/2, JAK/STAT. MAPK and PI3K/Akt are the two main pathways activated by the EGFR family, in particular the HER2 heterodimer (Figure 4). The activated MAPK pathway promotes transcription of related genes, subsequently enhancing cancer cell proliferation, migration, differentiation, angiogenesis and drug resistance. In the PI3K/Akt pathway, phosphorylated Akt acts on a range of transcription factors including MDM2, mTOR, p27, GSK3β, BAD, NF-κB, FKHR, enhancing proliferation, survival and suppressing apoptosis [12].
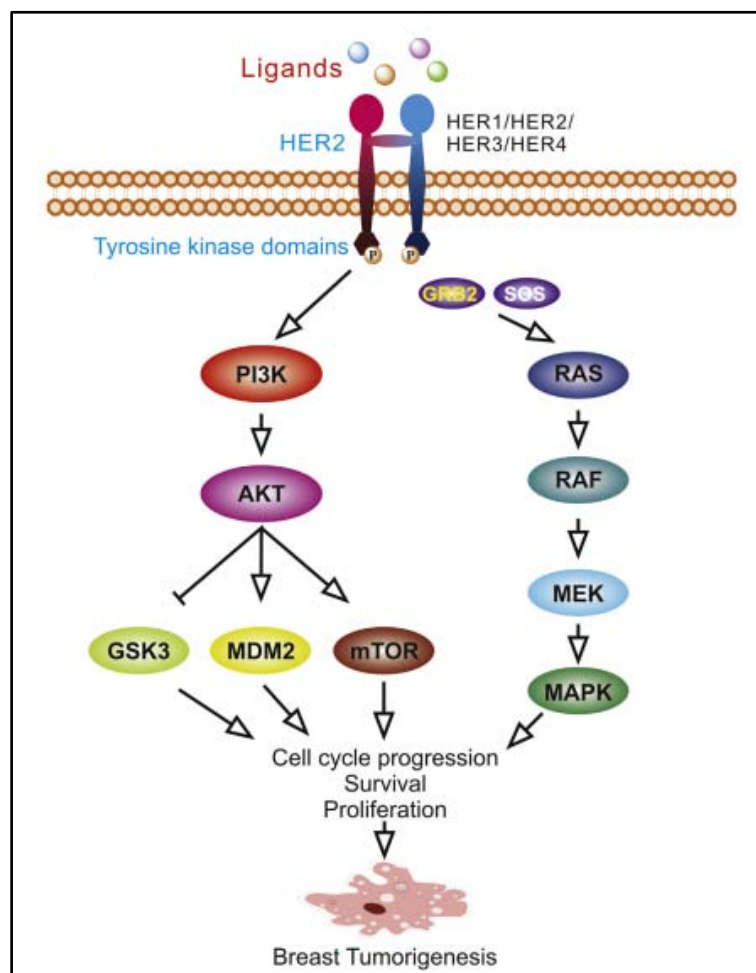
Figure 4: Phosphorylation of the tyrosine kinase domain of the HER2 receptor initiates downstream oncogenic signalling pathways, the main ones being the PI3K/AKT pathway and the Ras/MAPK pathway [1].

C/ Wnt/ β Catenin canonical signaling pathway

Wnt proteins are a family of highly glycosylated proteins that play an essential role in various developmental processes, including embryonic induction, generation of cell polarity as well as maintenance of adult tissue homeostasis. Canonical Wnt/β-catenin signaling is initiated by the binding of Wnt proteins to the two co-receptors Frizzled and Low-Density Lipoprotein Receptor-Related Protein 5 and 6 (LRP5/6). The Wnt/receptor interaction leads to the recruitment of Axin and Dishelved proteins to the cell membrane, and induces inhibition of protein glycogen synthase kinase (GSK)-3b. The latter is a negative regulator of the Wnt pathway, which leads to the degradation of β- catenin by the proteasome. Inhibition of GSK-3b leads to accumulation of β-catenin in the cytoplasm and its subsequent translocation into the nucleus acts as a transcriptional Co activator in synergy with other transcription factors such as T-cell factor/lymphoid enhancing factor (TCF/LEF). β-catenin regulates the transcription of several oncogenes, such as c-MYC, CCND1 and other target genes [13] (Figure 5).
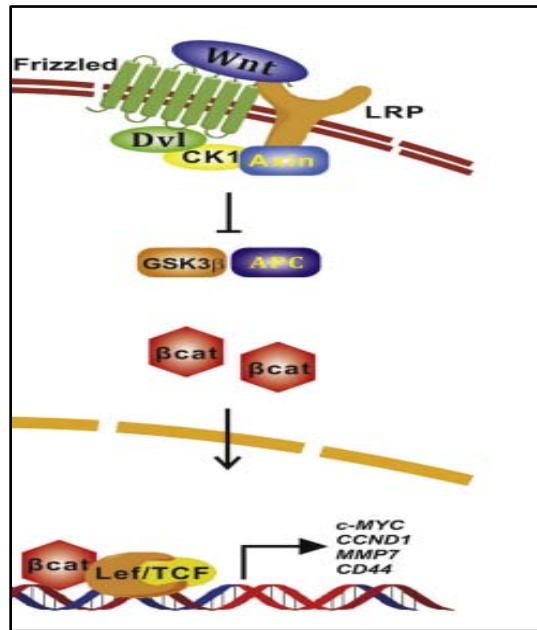
Figure 5: The Wnt ligand binds to frizzled membrane receptors and LRPs, leading to an accumulation of β-catenin in the intracytoplasm. Once β-catenin has been translocated to the nucleus, itactivates transcription of oncogenes [1].

D/ Notch signalling pathway

Since the cloning of the Notch gene and identification of the structure of this receptor in the 1980s, several studies have been accumulating to confirm the role of this pathway in physiological processes, essentially neuronal differentiation, mesoderm induction during embryogenesis and the choice of commitment to B or T lymphoid lineages during hematopoiesis. It is reactivated in the carcinogenesis of solid tumors: bronchial cancer, breast cancer, pancreatic cancer, melanoma...and hematological malignancies [14].

The Notch pathway comprises four transmembrane receptors, Notch1 to Notch4 (N1-N4), and five ligands: Jagged1 and 2 (Jag1-2), and Delta-like1, 3 and 4 (DLL1, 3 and 4). The extracellular region of the receptor comprises a ligand-interacting domain consisting of 29 to 36 EGF-like motifs, plus 3 cysteine-rich LNR (Lin12 and Notch Repeats) motifs that prevent receptor activation irrespective of the presence or absence of ligand. The transmembrane region contains a heterodimerization domain (HD). The cytoplasmic portion includes a RAM domain for binding to its transcriptional partner (RBPJκ), an ANK (Ankyrinrepeats) domain, two NLS nuclear localization signals, a TAD transactivation domain and a PEST domain on the C-terminal side, serving as a proteasomal degradation signal of the NICD [14](Figure 6).
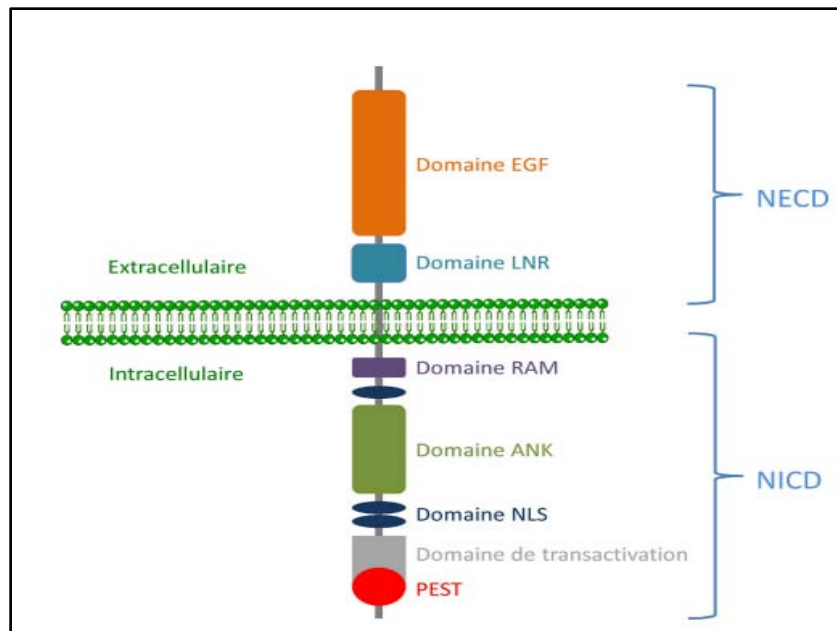
Figure 6: Schémas montrant les différents domaines constituant le récepteur de la voie NOTCH[14]

This signaling pathway is activated by the engagement of receptors with their ligands, expressed on a cell adjacent to the one receiving the signal. In the canonical pathway, ligand-receptor interaction leads to cleavage of the receptor, allowing release of its intracellular domain (NICD) and translocation to the nucleus. There, it associates with its transcriptional partner RBPJκ, which is DNA-bound to the promoter of the pathway's target genes (Figure 7). In the absence of NICD, RBPJκ is associated with transcriptional corepressors. Formation of the NICD-RBPJκ complex allows exclusion of these corepressors and recruitment of MAML (Master mind-like), which appears to serve as a scaffolding protein enabling formation of a transcriptional complex including other coactivators. This leads to chromatin opening and induction of transcription of target genes [15].
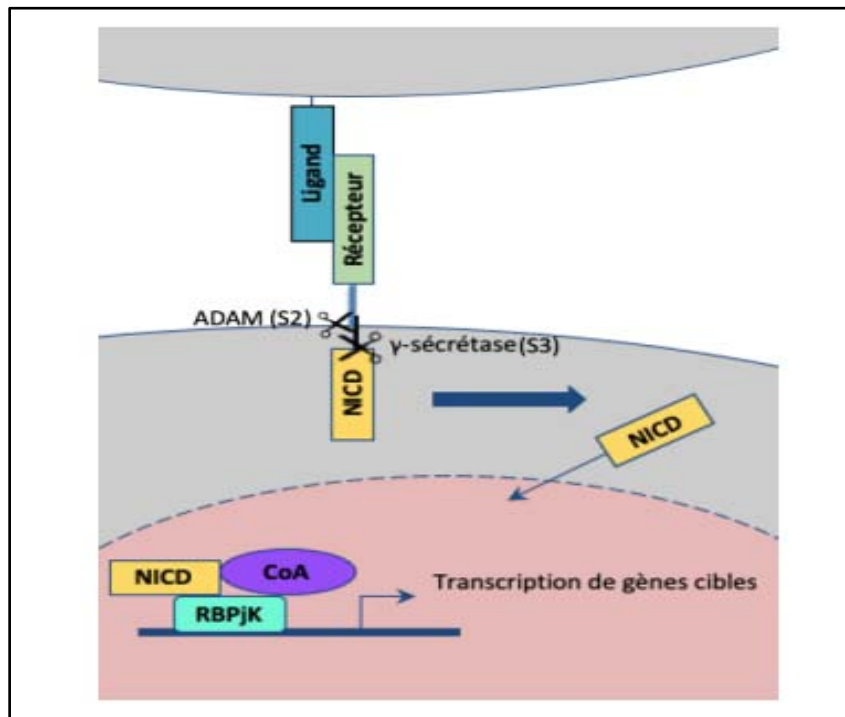
Figure 7: NOTCH signalling pathway: ligand-receptor interaction leads to cleavage of the Notch receptor, allowing release of its intracellular domain (NICD) and translocation to the nucleus. There it associates with its transcriptional partner RBPJκ, which is bound to DNA to induce transcription of target genes [15].

Notch signaling has several direct target genes involved in cell cycle regulation. These include cyclins A, B and D1, and members of the Hes/Hey family. It also activates major oncogenic signaling pathways such as c-Myc, Ras and Wnt [16].

Notch signaling inhibits breast cancer cell apoptosis through various signaling pathways [16] (Figure 8):

Activation of Akt signaling via NFκB, PI3K and mTOR signaling. Akt is responsible for direct inhibition of p53 or via the ASK1/JNK complex.

Activation of the c-Myc gene, which also has anti-apoptotic activity.

Upregulation of survival by blocking apoptosis via direct and indirect inhibition of caspases.

Upregulates anti-apoptotic members, notably Bcl-2 and Bcl-XL, while downregulating pro-apoptotic members such as Bim and Noxa.

Notch signaling reduces the sensitivity of cells in triple-negative TNBC breast cancer to TRAIL death receptor-induced apoptosis.

Stimulation of the synthesis of cyclin-dependent kinase inhibitors p21 and p15, which also contribute to resistance to apoptosis.
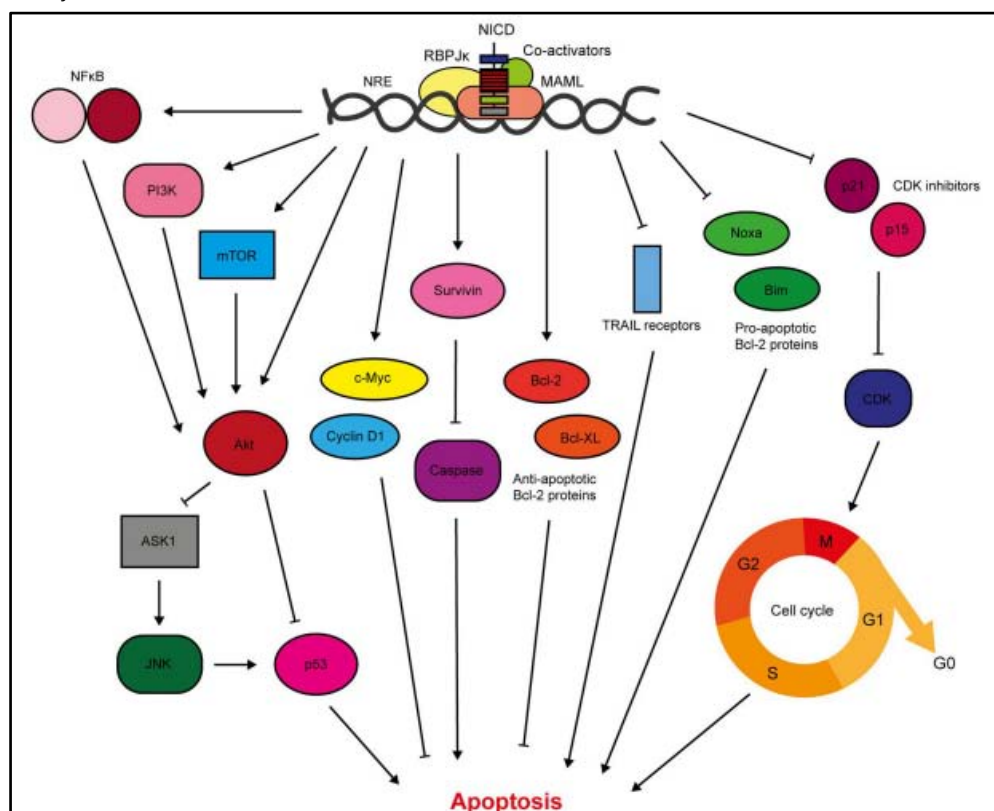
Figure 8: Diagram showing the role of the Notch pathway in regulating resistance to apoptosis [16].

Notch-mediated metastasis is induced primarily via TGFβ activation. It activates key regulators of the epithelial-mesenchymal transition EMT, notably the transcriptional repressors Slug and Snail, which mediate loss of cell-cell contacts through inhibition of E-cadherin expression. The mesenchymal markers ZEB1, β-catenin, N-cadherin and vimentin are upregulated by Notch signaling. Also involved in invasion, it upregulates matrix-degrading enzymes including matrix metalloproteinases 2 and 9 and urokinase-type plasminogen activator (uPA), as well as β1-integrin [16].

Notch signaling is aberrantly activated in breast cancer. Overexpression of Notch receptors and ligands has been correlated with a poorer prognosis: resistance to chemotherapy and early recurrence. The data suggest that deregulation of Notch signaling is an early

event in breast cancer tumorigenesis, with NICD accumulation in a wide range of subtypes, including ductal carcinoma in situ and epithelial hyperplasia. This implies that aberrant Notch signaling plays a causal role in breast tumor initiation [16].

Aberrant Notch activation may be secondary to mutations such as [16]:

Activating mutations in and around the PEST domain serving as a proteasomal degradation signal for the intracytoplasmic domain of the Notch1, 2 and 3 receptor;

Mutations disrupting the NLR, nuclear signaling motif, and heterodimerization domains;

Notch4 overexpression;

One cause of aberrant Notch signaling frequently found in breast cancer is the loss of the Numb protein. The Numb protein has long been known for its inhibitory role in the Notch signaling pathway. It opposes the Notch pathway by inhibiting recycling to the plasma membrane. It induces stabilization of the Notch ligand Delta-like 4 (Dll4) for degradation by lysosomes. NumB directly inhibits Notch by inducing polyubiquitination and preventing the activated intracytoplasmic domain from accessing the nucleus [17].

However, a recent Chinese study published in 2019 investigated the Notch pathway on nerve cells cultured in appropriate media, surprisingly showing that the NUMB protein enhances Notch signaling in a physiological way. In fact, the intracytoplasmic domain of the NOTCH type 1 receptor, N1ICD, undergoes various post-translational modifications, including ubiquitination by the BARD1-BRCA1 complex, facilitating degradation of the Notch receptor by the proteasome. The team discovered a new protein, BAP1, an enzyme capable of stabilizing N1ICD through its deubiquitination role and its ability to inhibit BRCA1. NUMB enhances Notch signalling by regulating the ubiquitin activity of the BAP1 protein and facilitating its association with N1ICD [17] (Figure 9).
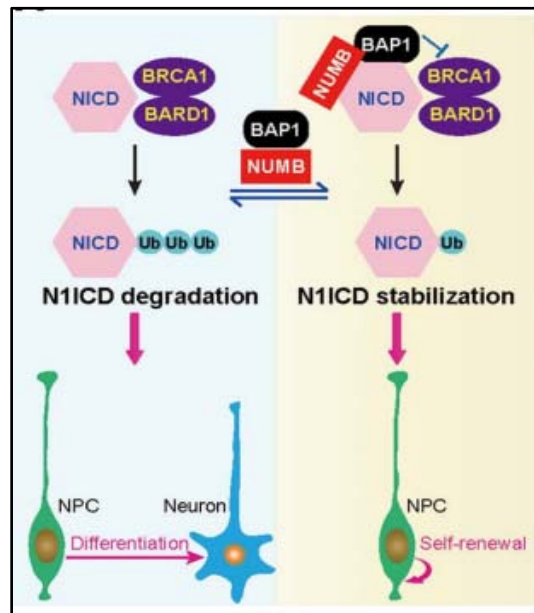
Figure 9: Schematic showing the role of NumB in stabilising N1ICD by associating with BAP1 to repress BRCA1/BARD1 ubiquitination activity in cortical neurons [17].

According to this new experiment, the importance long attributed to the Numb protein as a tumor suppressor in breast cancer is called into question. Explaining the relationship between loss of the Numb protein and breast cancer will be the subject of further exploration in the future.

E/ The sonichedgehog signaling pathway: SHH

The first identification of the sonichedgehog signalling pathway was made by EricWieschaus and Christiane Nüsslein-Volhard in 1980 during research on embryonic development in Drosophila melanogaster. This scientific paper won them the Nobel Prize for Physiology and Medicine in 1995.

Since its discovery, numerous studies have established the importance of SHH signaling in human embryogenesis and organogenesis, as well as its involvement in hematopoiesis. Normally, this pathway is inhibited in adults. However, it is reactivated in situations of tissue regeneration and stem cell renewal. Scientific research has identified three human homologues of the Drosophila hedgehog gene: sonichedgehog, deserthedgehog and indianhedgehog, of which sonic hedgehorg is the best-studied ligand. This complexification is reflected in its receptor protein patch homolog (PTCH), of which there are two in humans: PTCH1 and PTCH2. However, PTCH1 remains the most widely expressed receptor in human cells.

Once the Sonic hedgehog (SHH) protein has been synthesized by the cell, it acts autocrine or paracrine on the target cells. The SHH factor then interacts with its receptor: the protein patch homolog, PTCH1, located on the primary cilium. The ligand-receptor interaction triggers internalization of this complex into endosomal vesicles. This internalization lifts the repression on a receptor called protein smoothened SMO, initially located in intracellular vesicles and repressed by PATCH1. SMO will travel to the primary cilium, where it will modulate the complex containing the Suppressor of Fused (SUFU) protein and the inactive form of a protein called Glioma-associated, GLI. Dissociation of the SUFU-GLI complex leads to

degradation of the SUFU protein. In turn, the GLI factor undergoes transformations to acquire its active form. Activated GLI is translocated to the nucleus, where it specifically binds to sequences in the promoter regions of target genes, regulating their expression. These target genes include the GLI transcription factor itself, but also PTCH, cyclin D1 and products involved in the proliferation-differentiation balance (Figure 10). In the presence of the SHH ligand, the PTCH1 receptor's repression of the SMO protein is lifted. SMO then acts on the SUFU/GLI complex. SUFU is degraded, while GLI is activated and translocated to the nucleus. It acts as a transcription factor for several target genes, namely: PTCH1, GLI1, FOXA2, BCL-2, BCL-Xl, MYC and CYCLIN D1[18] (Figure10).
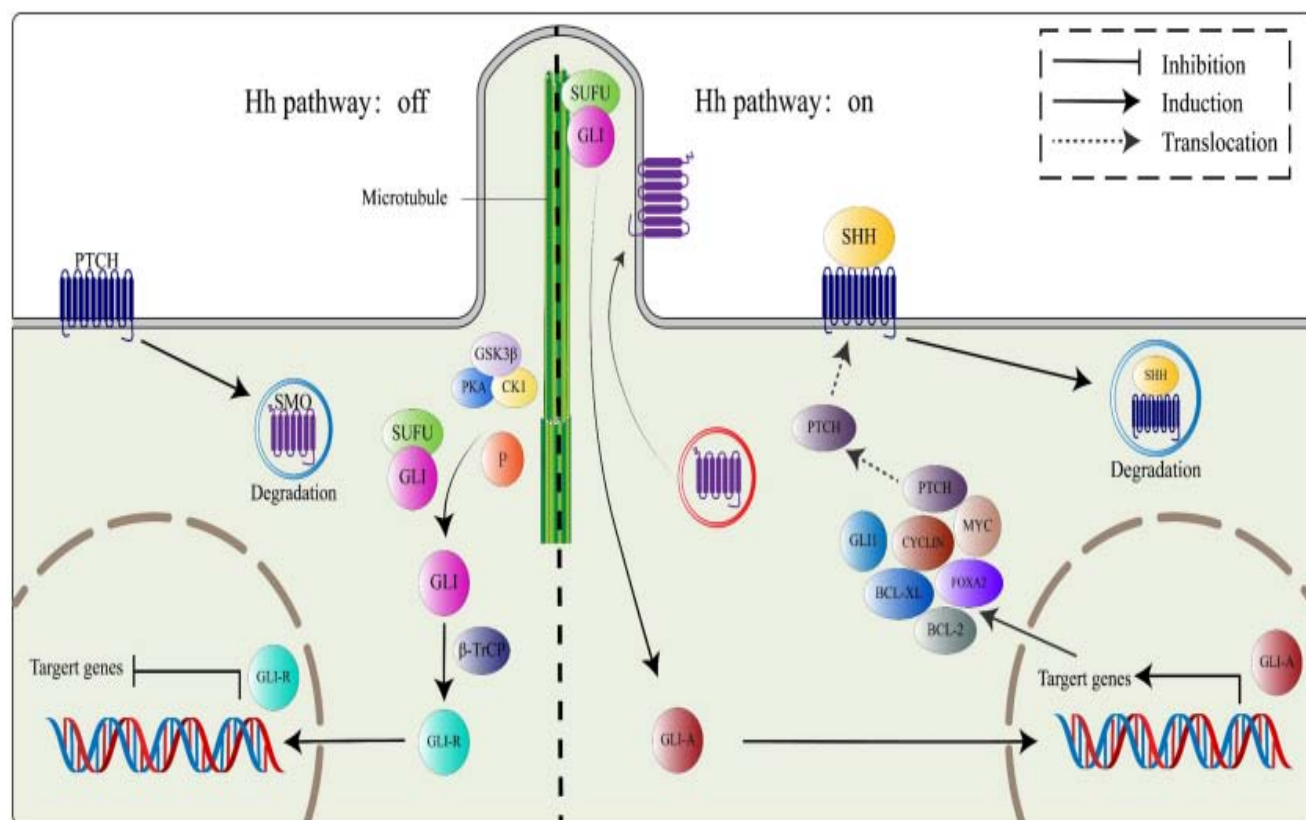
Figure 10: Diagram summarising the mechanism of action of the Sonic hedgehog SHH pathway: In the presence of the SHH ligand, the PTCH1 receptor lifts its repression of the SMO protein. SMO then acts on the SUFU/GLI complex. SUFU is degraded while GLI is activated and then translocated into the nucleus. IL acts as a transcription factor for several target genes, namely : PTCH1, GLI1, FOXA2, BCL-2, BCL-Xl, MYC and CYCLIN D1[18].

Activation of the HH signaling pathway in breast cancer has been associated with presentation at a younger age, larger tumor size, presence of lymph node metastasis, negative progesterone receptor status, high proliferation index and poor overall survival [19].

Mutations in SHH, PTCH1 and GLI1 are very rare in breast cancer. The pathological involvement of the SHH pathway is explained by several epigenetic mechanisms, the most important of which are:

The transcription factor NF-κB (nuclear factor-kappa B) positively regulates SHH protein expression. It has been shown that an NF-κB-binding element is normally present in a CpG island of the SHH promoter. This site becomes accessible to NF-κB binding after demethylation. Reduced CpG methylation of the SHH promoter has been linked to increased SHH expression in several cancers, including breast cancer.

Low expression of PATCH1, which acts as a negative regulator of HH signalling. This correlates with the hyper-methylation of its promoter. However, SHH can bind with high affinity to receptors other than PTCH1, such as PTCH2, HHIP, complicating the interpretation of experimental results aimed at elucidating the involvement of PTCH in breast cancer.

High levels of GLI1 expression are more common in triple-negative and basal-like breast cancers. Experimental studies have also revealed a GLI1 mRNA splicing variant responsible for the shorter, truncated form of GLI1 (tGLI1). This variant is capable of increasing the expression of GLI-selective target genes, such as VEGF-A, CD24, MMP-2 and MMP-9, inducing more invasive and pro-angiogenic forms of breast cancer with very high metastatic potential [20].

F/ Cyclin D-dependent kinase signalling pathway

Cyclin D1 amplification is observed in almost 60% of breast cancers. Estrogens also use cyclin D1 to exert their mitogenic effects. High cyclin D1 and HER2 overexpression have been reported to be associated with reduced recurrence-free survival and responsiveness to Tamoxifen [1].

G/ Mammary tumor kinase (BRK) signaling pathway

Also known as protein tyrosine kinase 6 (PTK6). It was originally cloned from a metastatic human breast tumor in 1994. The BRK transcript is encoded by an 8.93 kb DNA located on chromosome 20q13.3. The protein is a 451 amino acid kinase, comprising 3 parts, an SH3 domain and an SH2 domain, involved in protein-

protein interactions, and a tyrosine kinase domain (SH1). Compared with members of the Src family, BRK lacks an amino-terminal myristoylation sequence (a mechanism used to position proteins precisely in specific membrane compartments). The modification consists in adding a fatty acid called myristate to one end of a protein) which makes the protein soluble and accessible for interactions with intracellular substrates [21].

Breast tumor kinase is overexpressed in 86% of breast cancers [22]. The signaling events induced by PTK6 in the context of breast cancer are not well determined. However, it is clear that it is involved in several signaling pathways, summarized in figure 11.
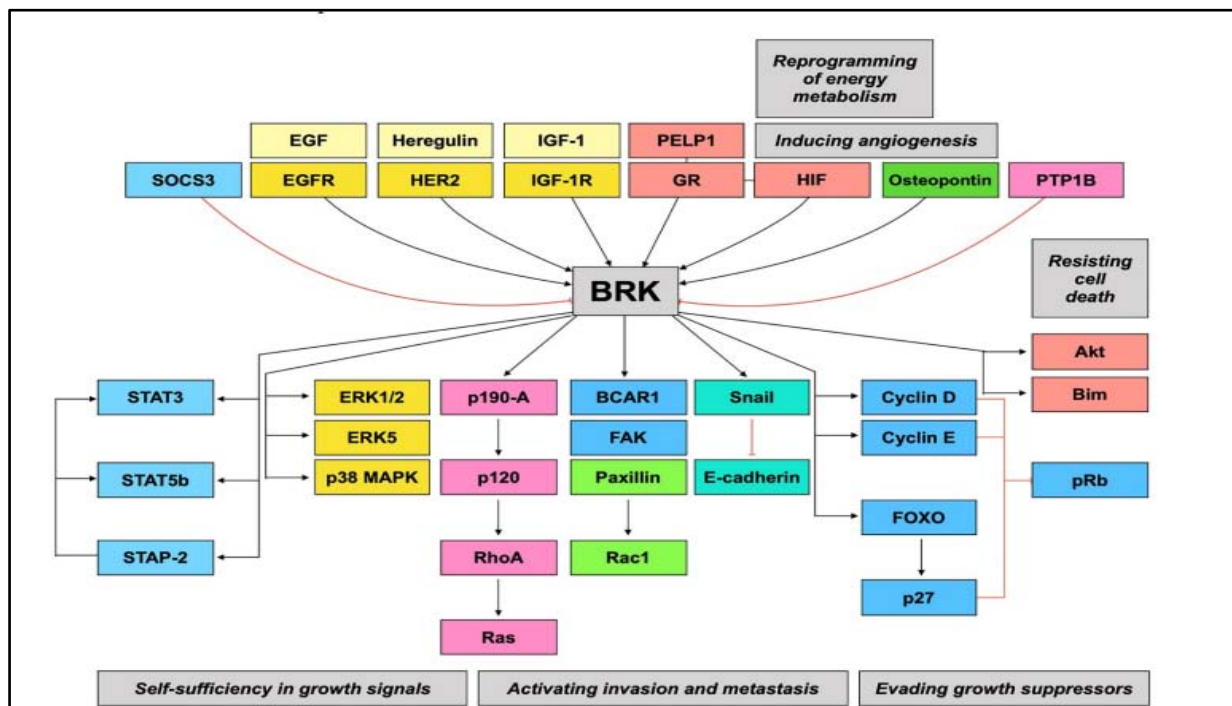
Figure 11: / The breast tumour kinase (BRK) signaling pathway is the convergence point for other signaling pathways that drive tumour progression [21].

H/ The PI3K/AKT/mTOR signaling pathway

Activation of the PI3K/AKT/mTOR signalling pathway is frequently found in breast cancer. The PI3PCA mutation is by far the most frequent mechanism, with mutation rates varying according to molecular subtype, with 49% of mutations found within luminal A, 32% within luminal B, 7% within basal-like, and 42% within HER2-enriched [23]. There are also numerous other mechanisms by which the PI3K signalling pathway is enhanced, such as HER2 amplification, IGF-1R overexpression, PTEN dysfunction and activating mutation of AKT1 [24].

Indeed, PI3P is made up of a catalytic subunit p110 and a regulatory subunit p85. There are three isoforms of p110, namely p110a (encoded by PIK3CA), p110b and p110d. PI3K signalling is most often initiated either by the tyrosine kinase of the growth factor-

activated receptor, or by the RAS protein, following a direct interaction with the p85 regulatory subunit, resulting in the recruitment of PI3K to the membrane [1]. PI3P assumes the phosphorylation of phosphatidylinositol-2- phosphate (PIP2) to phosphatidylinositol-3 phosphate (PIP3). This activating phosphorylation is finely regulated by the phosphatase PTEN (Phosphatase and tensinhomolog), whose role is to dephosphorylate PIP3 to PIP2. In this way, other downstream mediators are activated, AKT and mTOR leading to increased growth, translation, cell cycle progression and anti-apoptotic action [24].

The four main somatic mutations in PIK3CA are gain-of-function mutations involving four amino acids: E542K or E545K (glutamic acid at position 542/545 is replaced by lysine) located in exon 9, and H1047R (hestidine at position 1047 is replaced by arginine) or

H1047L (hestidine at position 1047 is replaced by leucine) located in exon 20. Mutations in exon 9 allow the p110α catalytic subunit to escape the inhibitory effect of p85. Mutations in exon 20 are located near the activation loop in the kinase domain. The mechanism by which they promote PI3K signaling is not well elucidated. [24].

Since its discovery in 2004, several studies have examined the prognostic and predictive value of PIK3CA gene mutations. One study showed that exon9 mutant patients were associated with a higher recurrence rate than exon20 mutant patients. [25]. Experimental and clinical evidence suggests that resistance to hormone therapy is largely due to hyperactivation of the phosphatidylinositol 3-kinase (PI3K) pathway. Breast cancers resistant to anti-estrogens often remain sensitive to hormone therapy combined with PI3K inhibitors [26] A 2018 analysis of 10329 patients with early-stage breast cancer found a significant association between early recurrence and PIK3CA mutations. In addition, PI3PCA mutations are predictive of poor response to anti-HER2 targeted therapy, with lower pCR (pathological complete response) rates than wild-type PI3PCA. [27].

IV. Hereditary Predisposition to Breast Cancer

15-20% of breast cancers run in families: patients with breast cancer have one or more first- or second-degree relatives with the disease. High-risk genes, accounting for around 20% of familial risk, are BRCA1, BRCA2, TP53, STK11, CD1 and PTEN. It should be noted that over 50% of the genetic inheritance of familial breast cancer remains uncertain [28].

A/ BRCA1/BRCA2 genes

In 1994, the BRCA1 gene was the first to be identified as a susceptibility gene for hereditary breast cancer. It is located on the long arm of chromosome 17 at 17q12-2. BRCA2 is located on chromosome 13, and was cloned in 1995[29]. Mutations in the BRCA1/BRCA2 genes are autosomal dominant. In the physiological state, BRCA proteins share a similar, cooperative tumor-suppressing mechanism by repairing DNA damage in double-strand breaks via homology-directed repair (HDR). Homologous recombination is based on faithful restoration of the damaged DNA sequence, using the homologous sequence of the undamaged chromosome as a template for repair. When this system is deficient, the relay is taken over by alternative DNA repair pathways that are much less genomically stable and considered mutagenic. This can lead to the activation of oncogenes or the inactivation of tumor suppressor genes, which explains the increased carcinogenic potential of these mutations [30]. However, homologous recombination primarily involves the detection of alterations by ATM (Ataxia telangiectasia mutated) and

ATR (Ataxia telangiectasia and RAD3-related) proteins and the mediation of signals by CHEK2 (checkpoint kinase 2) and BRCA1 itself. [29].

The Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA) has made significant contributions to the characterization of the BRCA landscape. A recent update report on the CIMBA dataset summarized a total of 1650 and 1731 unique mutations in BRCA1 and BRCA2 respectively [31] The most common type of mutation affecting these genes is the reading frame shift, leading mainly to the generation of premature stop codons and thus decreasing the levels of mature RNAs and functional proteins. [31].

Women with a BRCA1 mutation develop breast cancer with features similar to those found in the basal like non-hereditary subtype: young age (average 44 years), SBR grade III infiltrating ductal breast carcinoma (in 85% of cases), extensive lymphocytic infiltrate, foci of necrosis, a very high proliferation index, Ki67, and a triple-negative phenotype. A p53 gene mutation is found in 50 to 77% of cases, in contrast to sporadic forms, where it accounts for no more than 20%. Although BRCA1 mutant tumors are aggressive, they are more sensitive to cytotoxic agents, which improves their prognosis. In contrast, BRCA2 mutant tumors are heterogeneous, resembling sporadic tumors, with no dominant histological type, most often high-grade, and luminal in type. [29].

PARPs are enzymes involved in DNA repair for single-strand breaks. These repair pathways, via BRCA proteins (double-strand breaks) and PARP enzymes (single-strand breaks), are complementary: if one pathway is deficient and the other is blocked, the result is cell death by apoptosis, a phenomenon known as synthetic lethality. Single-strand breaks not repaired by PARP inhibition are converted into double-strand breaks during replication, unrepaired by the homologous repair system in the case of BRCA1 or BRCA2 mutation, leading to cell cycle arrest and apoptosis: this is known as double blockade (Figure 12). PARP inhibitors were first proposed and developed in ovarian cancer, then breast cancer, in cases of somatic or constitutional BRCA mutation, and more recently more widely, with significant positive results. The treatment received U.S. Food and Drug Administration (FDA) approval in the breast cancer indication in January 2018 [30].
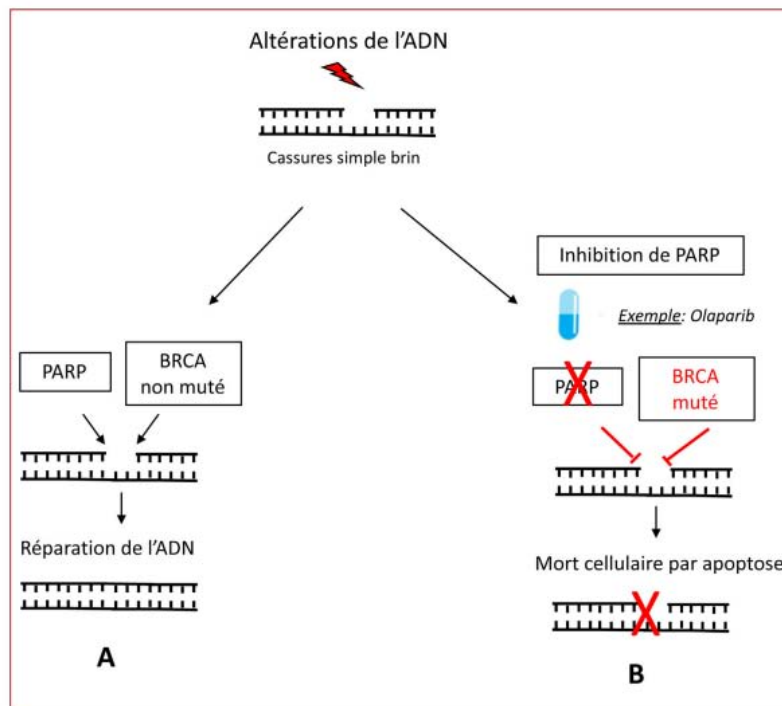
Figure 12: Schéma montrant le mode d'action des inhibiteurs de PARP: principe de létalité synthétique [30]

B/ The PALB2 gene

The PALB2 gene was discovered in 2009. This gene is responsible for Fonconi anemia in the case of a biallelic mutation. Located on the lower short end of chromosome 16 (16p12.2), it codes for a protein enabling interaction between BRCA1, BRCA2 and other proteins involved in the repair of double-strand breaks. Indeed, the aminoterminal end of the PALB2 protein interacts with BRCA1 and RAD51, while its carboxyterminal end features a WD40 domain enabling interaction with BRCA2 and polymerases [32]. Recently, several studies have shown that patients with mutations in the PALB2 gene are just as likely to develop breast cancer as patients with mutations in the BRCA1 and BRA2 genes. These patients develop breast cancer of predominantly triple-negative phenotype, with a significantly shorter life expectancy than BRCA1-mutated patients. [29].

C/ The PTEN gene

The PTEN gene is a tumor suppressor gene located on chromosome 10, 10q23.3. It codes for a phosphatase that dephosphorylates phosphatidylinositol-3- phosphate (PIP3) to phosphatidylinositol-2-phosphate (PIP2), thus antagonizing the action of PI3K (phosphatidylinositol-3-kinase). A loss-of-function germline mutation in PTEN is responsible for a syndromic inherited predisposition: Cowden syndrome, an autosomal dominant disorder with variable age-related penetrance. This syndrome is manifested by damage to tissues derived from all three embryonic lineages [29]. Clinically, patients with this syndrome present with macrocephaly, mucocutaneous lesions (trichilemmomas, papullomatous papulles, etc.), benign lesions such as hamartomas, lipomas or fibroids [33]. In certain situations, clinical diagnosis is difficult, requiring molecular confirmation to retain the diagnosis [29].

50% of patients with this syndrome develop breast cancer by the age of 40. The histological appearance is characteristic: apocrine ductal carcinoma, most often with solid architecture surrounded by hyalinized collagen, with moderate to high mitotic activity and positive androgen receptors. [29][33].

D/ The TP53 gene

A germline mutation in the TP53 gene constitutes li Fraumni syndrome. A second lesion affecting the second allele is required to initiate the process of carcinogenesis. Patients with this syndrome easily accumulate genetic abnormalities, and are at risk of developing cancers at a young age (< 30 years), or even during childhood. These patients may develop several cancers simultaneously. These include hard and soft tissue sarcomas (rhabdomyosarcomas), adrenocortical tumors, brain tumors and leukemias. [34]. Patients with this syndrome develop breast cancer in over 29% of cases, without it being a characteristic histo-morphological feature. Malignant phyllodes tumours are also frequent. Male breast cancer is rarely described in the context of Li Fraumeni syndrome. [33]. Because of the major carcinogenic effects induced either by therapy: chemotherapy, radiotherapy or

imaging-related irradiation, the latter present very restricted indications, hence the need to recognize this syndrome and confirm it by molecular study [33].

E/ The CDH1 gene

The CDH1 gene is located on the long arm of chromosome 16, 16q22.1. It codes for E-cadherin, a transmembrane intercellular adhesion protein involved in cell polarity and motility, as well as regulation of the microtubule network and organization of the actin cytoskeleton. A germline mutation in the CDH1 gene causes hereditary diffuse gastric cancer. Women with this mutation are at very high risk of developing lobular breast cancer, similar to that observed in women with BRCA1 or BRCA2 mutations. This cancer is observed either in the context of hereditary diffuse gastric cancer or without a family history of gastric cancer. Morphologically, lobular carcinoma developed in the context of a hereditary CDH1 mutation is similar to that observed in sporadic lobular carcinoma[29][33].

F/ The STK11 gene

Inactivation of the STK11 (Serine/Threonine Kinase 11) gene is responsible for Peutz-Jeghers syndrome, an autosomal dominant disease with complete penetrance. The incidence of this disease is estimated at one case per 100,000 births. This tumor suppressor gene is located on the short arm of chromosome 19, 19p13.3. It codes for a protein involved in the regulation of several cellular functions (such as signaling, apoptosis, cell polarity, regulation of the mTOR pathway), the most important of which is the limitation of cell proliferation in the event of restricted energy resources. Patients with Peutz-Jeghers syndrome clinically present with hyperpigmented macules on the oral mucosa, lips, palms and soles, as well as colonic and gastric hamartomatous polyps. These patients are at high risk of developing several types of cancer: colorectal, breast, small intestine, pancreatic, stomach and ovarian. In women with this mutation, the risk of developing breast cancer is around 15%, with a cumulative risk at age 70 of 45%.[29][34].

G/ The ATM gene

Germline mutation of the ATM gene, located on chromosome 11, is the cause of ataxia telangiectasia syndrome, an autosomal recessive disease with an estimated prevalence of 1 case per 40,000 to 100,000 births. These mutations can affect any region of the gene, and are of the following types: compound heterozygous nonsense mutations or a reading frame shift inducing deletions and insertions. This syndrome was first reported in 1957, and is characterized by progressive cerebellar ataxia revealed in early childhood, predominantly ocular telangiectasia, humoral immunodeficiency with low immunoglobulin production, and a predisposition to developing cancers, mainly lymphoid in children, as well as solid tumors, notably of the breast and stomach. The degree of inactivity of the ATM protein means that the clinical presentation is highly heterogeneous, and benign forms with late-onset symptoms have also been described. [35].

The ATM gene plays an important role in DNA double-strand break repair, apoptosis, cell cycle arrest and proliferation. It is also involved in the recombination of immunoglobulin chains and the TCR [34].

The risk of developing breast cancer in women with ataxia telangiectasia syndrome is very high, close to that of mutations affecting the BRCA genes. A recent study estimates that germline mutation of the ATM gene accounts for 1% of all women followed for breast cancer. All molecular subtypes are associated with this mutation, with the exception of triple-negative breast cancer [35].

H/ The BRIP1 gene

The BRIP1 (BRCA1-interacting protein C-terminal helicase 1) gene, located on chromosome 17, 17q23, codes for a protein belonging to the helicase family. It interacts with numerous other proteins involved in regulating responses to double-strand DNA damage, notably BRCA1, as well as checkpoint signaling during DNA replication. In 2006, truncated heterozygous germline mutations were identified in a hereditary context of breast cancer without BRCA1 and BRCA2 gene abnormalities. The relative risk of developing breast cancer in heterozygous women is estimated at two. This type of mutation results in overexpression of a short protein unable to interact with the BRCA1 protein.[36]. Homozygous mutations are responsible for Fanconi anemia [37]. However, another oncogenic role has been suggested in addition to tumor suppression. The BRIP1 gene modulates the expression of several other genes involved in growth. Deletion of BRIP1 mRNA leads to cell cycle arrest in the G1/S phase and reduced expression of genes: cMYC, Ras GTPase, and the Rb gene [38]. Several recent studies have comparatively assessed BRIP1 protein and mRNA expression levels in various cultured breast cancer cell lines versus normal control breast cells. The results show significantly elevated expression levels of this protein in various molecular subtypes of breast cancer. However, out of 1651 genes studied, no mutations in the BRIP1 gene were found. This further demonstrates the dual behavior of the BRIP1 gene. The same study highlights the role of BRIP1 in tumor invasion and metastasis. High levels of BRIP1 mRNA expression are associated with decreased expression of metalloproteases, down-regulation of the MGAT5 gene, involved in cell growth and motility, and the chemokine CXCL12, the only ligand for CXCR4, involved in the formation of the pre-metastatic niche. [38][39]. The BRIP1 gene may be a potential molecular biomarker for predicting the prognosis of breast cancer patients that can replace conventional prognostic and

analytical features such as lymph node status, tumor size, histological grade and molecular subtype [39].

Other genetic susceptibility genes for hereditary breast cancer include: NF1, NBN, CHEK2, RAD51C, RAD51D.

The various genes involved in hereditary breast cancer are currently the subject of genetic test kits (Oncotype DX and Mamaprint) whose published results are satisfactory for predicting the risk of breast cancer.

V. Conclusion

The phenomenon of dedifferentiation is a recently described complex phenomenon that joins the two older theories, stochastic and hierarchical, in explaining the origin of the breast cancer cell. Indeed, the stochastic theory explains the variability of breast tumour subtypes by the diversity of oncogenic events undergone by stem and/or progenitor cells; whereas, according to the hierarchical theory, the same oncogenic event can generate several tumour cell lineages following the normal cellular hierarchy. Indeed, this subject is still a research question, in the sense that specifying the subtype and determining the cell of origin is a mandatory step in developing personalized treatments. The main signaling pathways involved in tumorigenesis: the estrogen receptor pathway, HER2, Wnt/βcatenin, are the same ones that regulate normal breast development and mammary stem cells. Given that only 20% of breast cancers occur in patients with a family history of the disease, we deduce that the genetic abnormalities found in breast cancer essentially involve lifestyle risk factors.

Links of interest: The authors declare that they have no links of interest.

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Rhabdomyolysis in the Pathogenesis of Local Cold Injury

By M.I. Mikhailichenko, S.A. Figursky, V.A. Mudrov, V.V. Dorzheev,
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Введение- Современные классификационные градации отмороженийпредусматривают, что степени поражения варьируют от повреждения кожи, что свойственно дляI и II степени, до подкожно-жировой клетчаткиисубфасциальных поражений (в том числе мышц и костей) при III и IV степенях.Однако, необходимо указать, что даже поверхностные отморожения могут провоцировать затяжное и неблагоприятное течение постальтерационного периода с поражением мышечной и костной ткани. Причины вышеуказанных механизмовтребуют дальнейшего рассмотрения [1-4].

GJMR-C Classification: NLM: RC685.R4

RHABDOMYOLYSISINTHEPATHOGENESISOFLOCALCOLDINJURY

Strictly as per the compliance and regulations of:

Rhabdomyolysis in the Pathogenesis of Local Cold Injury

Рабдомиолиз в Патогенезе Местной Холодовой Травмы

M.I. Mikhailichenko [α], S.A. Figursky [σ], V.A. Mudrov [ρ], V.V. Dorzheev [ω], Yu.V. Mikhailichenko [¥] & K.G. Shapovalov [§]

I. Введение

Современные классификационные градации отморожений предусматривают, что степени поражения варьируют от повреждения кожи, что свойственно для I и II степени, до подкожно-жировой клетчатки и субфасциальных поражений (в том числе мышц и костей) при III и IV степенях. Однако, необходимо указать, что даже поверхностные отморожения могут провоцировать затяжное и неблагоприятное течение постальтерационного периода с поражением мышечной и костной ткани. Причины вышеуказанных механизмов требуют дальнейшего рассмотрения [1-4].

При воздействии холода в первую очередь страдают высоко дифференцированные ткани: эндотелий и нервная ткань [2, 3]. Однако, криоальтерации подвергается и мышечная ткань, которая, в свою очередь, составляет 40% массы тела человека. Альтерация мышечной ткани, или рабдомиолиз, сопровождается поступлением в кровоток продуктов распада миоцитов, таких как олиго- и полипептиды, лизосомальные ферменты, брадикинин, гистамин, серотонин и другие биологически активные вещества [5-7]. Необходимо отметить, что несмотря на многочисленные биологические эффекты указанных пептидов, последние не являются специфическими биомаркерами миоальтерации. Единственным биологическим активным веществом, подтверждающим деструкцию мышечной ткани, считается свободный миоглобин [8].

Огромное количество работ посвящено неблагоприятным кардиальным событиям, доказана роль миоглобина в формировании нарушений мозгового кровообращения, существенное внимание уделяется значению миоглобина при экзогенных травмах различной локализации [6]. Ученые приходят к единому мнению о значимой роли миоглобина в патогенезе любого повреждения, а также устанавливают существенные прогностические критерии в последствиях миоглобин-ассоциированной цитодеструкции.

Функциями миоглобина является депонирование и транспортирование кислорода к митохондриям внутри миоцитов [5-8]. Сам по себе свободный миоглобин токсичен. Он обладает сосудосуживающим действием, поражает эндотелий сосудов. При этом, учитывая его относительно большой объём, происходит закупорка канальцев почки, что в свою очередь ведет к развитию острой почечной недостаточности [9].

Определение концентрации миоглобина в крови используется при таких заболеваниях как дерматомиозит, полимиозит, у пациентов с генерализованной мышечной атрофией, газовой гангреной, лептоспирозом, краш-синдромом [7-9]. Однако работ, посвященных рабдомиолизу у пациентов с местной холодовой травмой, в современной литературе нет.

Цель работы: изучить уровень миоглобина и показатели биоэлектрической активности мышц у пациентов с местной холодовой травмой.

II. Материалы и Методы

В исследование включено 44 пациента (30 мужчин и 14 женщин) с отморожениями III–IV степени нижних конечностей. В зависимости от объема пораженных холодом тканей пациенты распределены на 3 группы: I группа-пациенты с поражением на уровне фаланг пальцев (n=14), II группа- на уровне плюсны (n=14), III группа поражение стопы и нижней трети голени (n=16). Учитывая сроки криоальтерации, уровень периферического миоглобина определялся на 5-е и 30-е сутки от момента получения травмы. В связи с этим пациенты были разделены на 2 группы: 1 группа – в позднем реактивном периоде (5-е сутки с момента травмы) (n=22), 2 группа – в периоде гранулирования и эпителизации (30-е сутки) (n=22).

Определение уровня периферического миоглобина выполнялось помощью мультиплексного анализа сыворотки крови набором реагентов фирмы Biomedical.

Для оценки биоэлектрической активности мышц использован неинвазивный метод накожной электронейромиографии. С помощью аппарата Нейро-ВМП, компании Нейрософт (г. Иваново) выполнялась электростимуляция накожным электродом позади

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медиальной лодыжки в углублении таранной кости. Оценивались амплитуда М-ответа и резидуальная латентность. Исследовалась функция мышц проксимальнее зоны демаркации.

Для определения состояния микроциркуляторного русла использован неинвазивный метод ЛДФ с помощью аппарата ЛАКК-02 (НПП «Лазма», Россия). Показатели ЛДФ регистрировались в течение 8 минут, фиксировались перфузионные единицы (пф.ед.). Датчик устанавливали по передней поверхности в средней трети голени. Оценивался показатель микроциркуляции (ПМ (пф.ед.)).

Контрольную группу составили относительно здоровые люди в возрасте от 27 до 40 лет (n=28).

Все пациенты и добровольцы, участвовавшие в исследовании, дали на это письменное добровольное информированное согласие. Работа выполнена в соответствии с требованиями Хельсинкской декларации Всемирной медицинской ассоциации (в ред. 2013 г.).

III. Статистический Анализ

При проведении статистического анализа авторы руководствовались принципами Международного комитета редакторов медицинских журналов (ICMJE) и рекомендациями «Статистический анализ и методы в публикуемой литературе» (SAMPL) [12, 13]. Анализ нормальности распределения признаков, с учетом численности исследуемых групп равной менее 50 человек, проводился путем оценки критерия Шапиро-Уилка. Учитывая распределение признаков, отличное от нормального во всех исследуемых группах, полученные данные представлены в виде медианы, первого и третьего квартилей: Me [Q_1; Q_3]. Для сравнения двух независимых групп по одному количественному признаку использовался критерий Манна-Уитни. Для сравнения количественных показателей зависимых исследуемых групп использовали ранговый критерий Уилкоксона, который в заключении SPSS автоматически преобразуется в величину Z (z-score). Во всех случаях p<0,05 считали статистически значимым. Статистическая обработка результатов исследования осуществлялась с помощью пакета программ «IBM SPSS StatisticsVersion 25.0» (International Business Machines Corporation, США).

IV. Полученные Результаты и их Обсуждение

Установлено, что на 5-е сутки с момента криоповреждения концентрация миоглобина в крови была выше в 35,6 [34,1; 57,5] раз (p<0,001) по сравнению с группой контроля. В тоже время отмечается меньшее значение амплитуды М-ответа – в 5,0 [4,4; 5,3] раз (p<0,001), большая резидуальная латентность – в 1,7 [1,6; 1,8] раза (p<0,001), показатель микроциркуляции меньше в 1,6 [1,5; 1,7] раза (p<0,001). На 30-е сутки уровень миоглобина выше в 7,0 [6,7; 8,3] раз (p<0,001) по сравнению с группой контроля, амплитуда М-ответа ниже в 1,8 [1,7; 2,1] раза (p<0,001), резидуальная латентность – выше в 1,5 [1,4; 1,5] раза (p<0,001), значение показателя микроциркуляции – ниже в 1,3 [1,2; 1,3] раза (p<0,001), что свидетельствует о статистической значимости различий указанных показателей в исследуемых группах (табл. 1).

Таблица 1: Сравнительный анализ уровня исследуемых показателей в исследуемых группах

Уровень исследуемых показателей	Исследуемые группы		Группа контроля, n=28	Тестовая статистика	
	1 группа, n=22	2 группа, n=22		Манна-Уитни	Величина Z
Время с момента криотравмы	**5-е сутки**	**30-е сутки**			
Уровень миоглобина,ng/ml	3133,2 [3133,2; 4774,3]	618,6 [617,8; 692,7]	88,0 [83,0; 92,0]	$U_{к-1}$=0,0, $p_{к-1}$<0,001; $U_{к-2}$=10,0, $p_{к-2}$<0,001;	Z_{1-2}=-4,01, p_{1-2}<0,001
Амплитуда М-ответа, мС	0,7 [0,7; 0,8]	1,9 [1,8; 2,0]	3,5 [3,5; 3,7]	$U_{к-1}$=0,0, $p_{к-1}$<0,001; $U_{к-2}$=0,0, $p_{к-2}$<0,001;	Z_{1-2}=-3,93, p_{1-2}<0,001
Резидуальная латентность, мС	5,4 [5,3; 5,5]	4,6 [4,5; 4,7]	3,1 [3,1; 3,3]	$U_{к-1}$=0,0, $p_{к-1}$<0,001; $U_{к-2}$=0,0, $p_{к-2}$<0,001;	Z_{1-2}=-3,89, p_{1-2}<0,001
Показатель микроциркуляции, пф.ед.	27,0 [26,3; 28,0]	33,0 [32,7; 35,6]	42,5 [42,2; 43,4]	$U_{к-1}$=0,0, $p_{к-1}$<0,001; $U_{к-2}$=10,5, $p_{к-2}$<0,001;	Z_{1-2}=-3,87, p_{1-2}<0,001

Примечание:

U – критерий Манна-Уитни, позволяющий попарно сравнить две исследуемые группы с группой контроля;

Z – величина Z (z-score), позволяющая оценить изменение уровня исследуемых показателей в динамике (сравнить 1 и 2 группу настоящего исследования);

p – значимость различий сравниваемых показателей.

На тридцатые сутки с момента криоповреждения отмечается снижение как уровня миоглобина, так и резидуальной латентности (p<0,001). Амплитуда М-ответа и показатель микроциркуляции, напротив, повышаются на тридцатые сутки в сравнении с пятыми сутками криоповреждения (p<0,001), что свидетельствует о преобладании репаративных процессов (табл. 1).

В зависимости от объема пораженных холодом тканей отмечается снижения показателей биоэлектрической активности мышц и повышение уровня периферического миоглобина. Так у пациентов I группы показатели амплитуды М-ответа снижались в 3 раза (p<0,001), между тем уровень миоглобина повышался в 3 раза (p<0,001),в сравнении с контрольной группой, у пациентов II группы данные показатели снижались в 4,8 раз (p<0,001), показатель миоглобина повышался в 6 раз (p<0,001, в III группе показатели снижались в 7 раз (p<0,001), уровень миоглобина повышался в 38 раз (p<0,001).Что касается резидуальной латентности, ее показатели повышались. У пациентов I группы резидуальная латентность в 1,4 раза (p<0,001) выше в сравнении с контрольной группой, у пациентов II группы - в 1,7 раза (p<0,001), и у пациентов III группы - в 2,4 раза (p<0,001) по сравнению с группой контроля (табл.2).

Таблица 2: Уровень миоглобина, биоэлектрической активности мышц, показателей микроциркуляции у пациентов с местной холодовой в зависимости от объёма поражения

Показатель	Группа контроля	I группа	II группа	III группа
Уровень МВ (ng/ml)	93,53 [73,55; 137,77]	272.73 [188,20;367,44] (p<0,001)	560.52 [401,95;853,91] (p<0,001)	3 556.61 [1761,71;14748,24] (p<0,001) (p1<0,001)
Амплитуда М-ответа (мС)	3,5 [3,37; 3,67]	1,16 [1,00; 1,30] p<0,001	0,74 [0,60; 0,90] p<0,001	0,53 [0,40; 0,63] p<0,001; p1<0,001; p2<0,001
Резидуальная латентность (мС)	3,2 [3,13; 3,32]	4,48 [4,18; 4,83] p<0,001	5,48 [5,28; 5,73] p<0,001; p1<0,001	7,56 [7,3; 7,8] p<0,001; p1<0,001; p2<0,001
Показатель микроциркуляции (пф.ед.)	5,92 [5,24; 6,72]	5,01[3,35; 6,87] p>0,05	2,93[2,31; 3,1] p<0,05; p1<0,05	6,99[4,32; 9,37] p<0,05 p1<0,05p2<0,05

Примечание:

p – достоверность разницы показателей относительно контроля;

p1 – достоверность разницы показателей относительно 1-й группы больных;

p2 – достоверность разницы показателей относительно 2-й группы больных.

Специфическим биологическим маркером, указывающим на нарушение целостности мембраны и альтерацию миоцитов, является свободный миоглобин [6, 7, 8]. Доказано, что его функция - депонирование и транспорт кислорода к митохондриям. Мощнейшаяэкзоцитическаятоксичность миоглобина обусловлена сосудосуживающим действием, а также альтерацией эндотелия. Необходимо указать и на негативную функцию свободных форм периферического миоглобина. Его участие в повреждении и закупорке почечных канальцев очевидна[5]. В наших более ранних работах описанахроническая почечная дисфункции у пациентов, перенесших местную холодовую травму [4].

У пациентов с местной холодовой травмой в реактивном периоде происходит поступление в кровеносное русло большого количества миоглобина (табл. 1). Наряду с этим, запредельные значения миоглобина фиксируются у пациентов с самыми обширными поражениями, уровень миоглобина почти в 40 раз превышает контрольные показатели (табл. 2). У свободного миоглобина сродство к кислороду гораздо выше, чем у гемоглобина[…]. Причем механизмы передачи молекул кислорода у свободного миоглобина полностью отсутствуют. Вероятно, что в очаге альтерации реализуется синдром обкрадывания тканей ввиду механического выключения периферического кровотока и отсутствие должной оксигенации тканей.

Формируется стойкая гипоксия в очаге альтерации. Последняя усугубляет повреждение и дисфункцию эндотелия, а также формирует стойкую гипоксическую полипатию в очаге поражения и перифокально.

Особенности и биологические эффекты нейродеструкции описаны в наших более ранних работах [2, 3]. Доказана существенная роль периферической нейропатии в формировании неблагоприятных последствиях криотравмы. Возможно, именно свободный миоглобин является первоисточником периферической гипоксической нейропатии ввиду его двойственной природы альтерации при местной холодовой травме. Тканевой дефицит кислорода ведет и к снижению тромборезистентности и увеличению адгезивной активности тромбоцитов и лейкоцитов [2, 3, 5].

Открытый в 1999 году профессором Ю.А. Витковским феномен лимфоцитарно-тромбоцитарной коагрегации считают вынужденным и обоснованным механизмом стабилизации эндогенной катастрофы [1]. Однако, полученные нами данные (снижение показателя микроциркуляции и высокие цифры миоглобина) в поздние сроки криотравмы заставляют задуматься и о негативном влиянии феномена ЛТА.

Помимо вышеуказанного, анатомо-физиологические особенностипоперечно-полосатой мышечной ткани, а также замкнутые фасциальные пространства, делают эти высоко дифференцированные

и относительно устойчивые к гипоксии ткани уязвимыми перед «синдромом замкнутых пространств». Доказано, что при ходовом повреждении миоцитов продукты их распада, такие как электролиты, лактатдегидрогеназа, брадикинин, серотонин и другие биологически активные вещества поступают в кровяное русло, вызывая выраженную эндотелиальную дисфункцию и активацию тромбоцитарного гемостаза [10]. Помимо этого, патогенетические механизмы, лежащие в основе рабдомиолиза и его последствий для пострадавших обусловлены двумя основными инициирующими механизмами – снижением энергообеспечения перифокальных миоцитов и образованием активных форм кислорода, приводящими к выраженной митохондриальной дисфункции с активацией апоптоза и последующей цитодегенерацией [4, 5]. Высвобождение внутриклеточного содержимого вызывает локальное повреждение капилляров, секвестрацию жидкости во внеклеточном пространстве поврежденных мышц с последующей системной гиповолемией, гиперкалиемией, ишемией, метаболическим ацидозом и различными осложнениями [1–3]. Следовательно, описанные выше патофизиологические механизмы – составляющие затяжного и неблагоприятного течения криотравмы. Установлено, что и на 30 сутки криоповреждения выявляются высокие цифры миоглобина в крови пострадавших. Помимо вышеуказанного, миоглобин потенцирует повышение тонуса гладкомышечных волокон, входящих в состав сосудистой стенки, провоцируя эндотелий-независимую вазоконстрикцию и еще большее нарушение локальной гемодинамики [10, 11].

Эти явления подтверждаются полученными лабораторными и инструментальными данными: снижается амплитуда М-ответа, увеличивается резидуальная латентность, снижается показатель микроциркуляции у пострадавших. Вне всякого сомнения, продукты деградации миоцитов формируют и острое повреждение почек у пострадавших [4, 5].

Выявленные нами данные указывают на значительную, а порой, и главенствующую, роль свободного миоглобина у пациентов с отморожениями. Вероятно, повреждающее влияние последнего носит системный характер. Мы видим и почечное повреждение у пострадавших, и длительное течение процессов репарации, и относительнонеблагоприятные исходы даже при незначительном повреждении тканей при отморожениях. В настоящее время не существует специфических критериев связывания свободного миоглобина, а патофизиологическиемеханизмы развития этого синдрома разнообразны и, к сожалению, в большинстве случаев предполагаемы.

В связи с этим, разработка новых подходов к комплексной терапии отморожений, как многогранного и сложнейшего патогенетического процесса, а также механизмы инактивации свободного миоглобина выведут на новый уровень лечения отморожений и их неблагоприятных последствий, а также прочих тяжелых и жизнеугрожающих состояний.

V. Выводы

1. У пациентов с глубокими отморожениями происходит резкое повышение уровня миоглобина в ранние сроки криотравмы, при этом миоглобинемия сохраняется в отдаленные сроки травмы.
2. Высокая концентрация свободного миоглобина сопровождается нарушениями микроциркуляции и формированием локальной нейропатии.
3. У пациентов с отморожениями снижается амплитуда М-ответа и повышается резидуальная латентность.

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The Regeneration and Growth of Planaria: A Study Testing Planaria Regeneration Effectiveness in Different Solutions

By Rosangela Le, Leul Wondwossen & Dr. Patanarut

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Abstract- The Regeneration and Growth of Planaria: A Study Testing Planarian Regeneration Effectiveness in Different Solutions.

Planarian regeneration can be affected by its environment. Planaria can regenerate, making them perfect to compare regenerative capacity of variables. The quality of what the planaria live in can affect how well they regenerate. Planaria can regenerate quickly due to their size and simplicity, allowing for results to be revealed quickly. In this experiment, planaria will be vertically cut in half, then placed in jars with different solutions. One jar will contain spring water (planaria's normal habitat). Three other jars will be filled with 15% green tea, and caffeine solutions and then a 5% creatine solution. The jar with spring water will be the control group.

GJMR-C Classification: LCC: QH301

THEREGENERATIONANDGROWTHOFPLANARIAASTUDYTESTINGPLANARIAREGENERATIONEFFECTIVENESSINDIFFERENTSOLUTIONS

Strictly as per the compliance and regulations of:

The Regeneration and Growth of Planaria: A Study Testing Planaria Regeneration Effectiveness in Different Solutions

Rosangela Le [α], Leul Wondwossen [σ] & Dr. Patanarut [ρ]

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Planarian regeneration can be affected by its environment. Planaria can regenerate, making them perfect to compare regenerative capacity of variables. The quality of what the planaria live in can affect how well they regenerate. Planaria can regenerate quickly due to their size and simplicity, allowing for results to be revealed quickly. In this experiment, planaria will be vertically cut in half, then placed in jars with different solutions. One jar will contain spring water (planaria's normal habitat). Three other jars will be filled with 15% green tea, and caffeine solutions and then a 5% creatine solution. The jar with spring water will be the control group. Three trials testing planarian regeneration will be done. Pictures of planaria during trials will be taken and results of the regeneration from each jar will be compared. Planaria will be placed in each jar for one trial. Planaria growth will be observed using a stereoscope and camera. Planaria will not be reused for the other trials. The planaria's manipulated environment should influence their regeneration. The purpose of this experiment will be to determine the regeneration capacity of the variables we use on the Planaria in hopes that the regenerative features can be used in different medicines in the long term.

I. CHAPTER ONE

a) Introduction

Planaria are known for having amazing regenerative abilities. Scientists have studied planaria before in hopes of finding ways to apply planarian regenerative abilities to humans. Planaria are able to fully regenerate even when they are split in half. When cut into a tiny piece, planaria can grow the rest of their body back. The purpose of this research project is to find what most positively impacts the regeneration of planaria. Because they have such phenomenal regeneration capabilities, planaria have often been studied in hopes of finding links for regeneration in other species. Studying planaria regeneration helps us better understand how it works and can potentially be used towards regeneration in humans. During a study done on neural regeneration, researchers looked into regenerating the optic nerve using peripheral nerve tissue. They were able to take the peripheral nerve tissue that stemmed from the spinal cord down the leg and used it to replace the optic nerve. Science is currently able to replace the nerves of tissue. The thing about nerves is that some of them (specifically the ones connected to the central nervous system) specify with a certain function and cannot be replaced by just any random nerve. Due to this, not all nerves can be replaced, and those nerves are crucial which in turn makes neural regeneration so important. Looking into stem cells, they noticed their ability to regenerate any type of cell. The main problem being that the cells they regenerate are still more of the basic standard cells that are not specific in their function but rather just go with the rest of the single function cells. Better understanding regeneration can greatly benefit by helping people recover from major injuries. Studying what positively effects planaria regeneration builds a guideline for what to continue looking for future research. The question being studied is "Which planarian will regenerate with the best quality when kept in regular spring water, creatine solution, caffeine solution, and green tea solution?" It is hypothesized that after being split vertically in half, planarian in the creatine solution will grow the best compared to planarian kept in the other solutions.

II. CHAPTER TWO

a) Materials
- 120 black planarian
- Scalpel
- Petri dishes
- 2 gallon of spring water
- 5.1 oz creatinol serum
- 240 mL liquid caffeine
- 16 oz bottle of unsweetened green tea
- Disposable pipettes
- Stereomicroscope
- Distilled water
- Phone camera
- Hard-boiled egg yolk
- Ruler

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b) *Procedures*

1. Prep the creatine, caffeine, and green tea solution in 3 different beakers. Label the beakers so the solutions are not mixed up. The creatine should be made with a 5% concentration while the caffeine and green tea solution will be 15%. Keep the planaria in the jar they came in but replace the water every couple days to keep it fresh. Make sure the planaria are well fed before starting the trials.

2. Fill nine petri dishes with the solutions using a pipette. Each solution should have three petri dishes. Three other petri dishes should be prepared, but with spring water. Make sure the pipettes are only used for one solution and do not touch the lab bench. Everything needs to be as sterile as possible to prevent any chances of the planaria dying.

3. Turn on the stereoscope and use the second light setting. Place a planaria on the lid of the petri dish with some water, then place the lid on the stereoscope. Using a phone camera, record the planaria next to a ruler for scale. This allows for the full length of the planaria to be measured as planaria are able to bunch up to an extremely small size, then expand to a larger length. Watch the video and wait for the frame that shows the planaria at its full extended length. Record the measurement.

4. Once the original length is recorded, use the scalpel to split the planaria horizontally in half. One piece should be the head while the other piece is the tail. Using the phone camera, record the planaria with the ruler next to it. Watch the video and freeze at the frame that shows the planaria at its extended length, then record the measurements.

5. Place the two planaria pieces into the petri dish with the creatine solution. Repeat steps 3-5 until the other eleven petri dishes have planaria in them. Twelve planaria from the jar should be used, and the petri dishes should have 21 total planaria pieces in them.

6. After the trial is over, create a 10% bleach solution to dump the planaria in. This will kill them painlessly and disintegrate them so there is not solid waste. Once killed, dump the bleach solution down the drain. Clean all glassware thoroughly with soap. The stereoscope should be wiped down. Dispose the pipettes in the trash can.

7. Repeat steps 2-6 for the next two trials.

c) *Planaria Maintenance*

Planaria will be fed once a week with egg yolk. A fresh petri dish with egg yolk should be prepared. Planaria will be transferred to this petri dish with a pipette. Planaria will be left with the food for only thirty minutes. They should not be left with the food for any longer than that. In that time, a fresh environment with new solutions will be prepped for the planaria. After they are done feeding, the planaria will be moved to these new petri dishes. Air should be blown into the petri dishes once a week.

d) *Data Methodology*

Pictures of planaria will be taken twice a week during each trial. A ruler will be placed next to the planaria for scale and a phone camera will be used to take the picture. Pictures of the planaria in the jar and under the stereoscope will be taken. Planaria will be compared by looking at the quality they grew back in and the size. A rough estimate of their size will be guessed based on their length next to the ruler. The focus of this research is more on the quality of the planaria growth, rather than the length, so exact measurements of the planaria will not be needed. The estimated length of each planaria group will be averaged, then compared using the t-test. The p-value will also be calculated to determine the random chance that will prove the hypothesis true.

III. Chapter Three

a) *Data*

Before starting the trials, the lethal dosage was tested for each solution. Planaria were found dead in the 10% creatine solution, therefore making 5% their lethal dosage. Planaria were able to survive in 15% solutions of both green tea and caffeine. Planaria were measured before being split, then measured after being split in half. The length of the planaria were measured on Tuesdays and Thursday.

Pre-Trial Measurements

Creatine	Caffeine	Green Tea
1.45 cm	1.50 cm	1.75 cm
2.10 cm	1.45 cm	1.45 cm
1.70 cm	2.00 cm	1.10 cm

Figure 1: Three planaria were placed in each solution. Planaria were measured for their original length before being split. This table shows the measurements for each planaria for the first trial

Creatine	Caffeine	Green Tea
2.00 cm	1.60 cm	1.80 cm
1.80 cm	1.50 cm	1.55 cm
1.75 cm	1.50 cm	1.15 cm

Figure 2: This table shows the measurements for each planaria for the second trial

	P1	P2	P3
Spring Water	1.60 cm	1.40 cm	1.50 cm

Figure 3: This table shows the recorded original Lengths of the three planaria used as control in the spring water for the first trial

	P1	P2	P3
Spring Water	1.50 cm	1.25 cm	1.40 cm

Figure 4: This table shows the original planaria length in spring water as the control for the second trial

Creatine	Caffeine	Green Tea
1.30 cm	1.2 cm	1.10 cm
0.95 cm	0.85 cm	0.90 cm
0.60 cm	0.60 cm	0.50 cm
0.75 cm	0.80 cm	0.50 cm
0.90 cm	0.60 cm	0.65 cm
0.80 cm	0.70 cm	0.30 cm

Figure 5: The planaria were split into 6 pieces. Each piece was measured prior to being placed in the different solutions. This table shows the measurements for the first trial

Creatine	Caffeine	Green Tea
1.10 cm	0.70 cm	0.80 cm
0.50 cm	0.90 cm	0.90 cm
1.00 cm	0.70 cm	0.60 cm
0.60 cm	0.80 cm	1.00 cm
0.80 cm	0.70 cm	0.90 cm
0.70 cm	0.75 cm	0,45 cm

Figure 6: This table shows the measurements for the planaria in the second trial. The original planaria were split into a total of 6 pieces.

First Week Measurements

Creatine	Caffeine	Green Tea
0.90 cm	0.80 cm	0.60 cm
0.70 cm	0.75 cm	0.60 cm
0.80 cm	0.85 cm	0.70 cm
1.35 cm	0.90 cm	0.55 cm
1.00 cm	0.80 cm	0.95 cm
1.10 cm	0.80 cm	1.10 cm

Figure 7: The length of the planaria were measured two days after being split. This table shows data for the first trial

Creatine	Caffeine	Green Tea
1.10 cm	0.90 cm	1.00 cm
0.85 cm	0.70 cm	1.10 cm
1.20 cm	0.75 cm	0.70 cm
0.95 cm	0.80 cm	0.90 cm
0.70 cm	0.70 cm	0.50 cm
0.75 cm	0.75 cm	1.00 cm

Figure 8: This table shows planaria length after two days for the second trial

Planaria in the creatine solution were hyperactive and moving much quicker than the planaria in the other solutions. They were much more active than the other planaria. Planaria in the green tea solution were moving a little slower than usual. They were thought to be dead at first but started moving around. Planaria in the caffeine and spring water solution were moving at a normal pace.

Second Week Measurements

Creatine	Caffeine	Green Tea
1.30 cm	0.80 cm	0.70 cm
1.10 cm	0.50 cm	0.60 cm
0.90 cm	0.80 cm	1.10 cm
0.85 cm	1.10 cm	0.95 cm
1.40 cm	0.90 cm	0.60 cm
1.00 cm	0.95 cm	0.70 cm

Figure 9: The length of the planaria were measured seven days after the second measurements. This table shows the measurements for the first trial

Creatine	Caffeine	Green Tea
0.80 cm	0.75 cm	0.60 cm
1.00 cm	0.80 cm	0.70 cm
1.50 cm	1.00 cm	1.00 cm
0.90 cm	0.95 cm	1.10 cm
0.80 cm	0.85 cm	1.00 cm
1.10 cm	0.80 cm	1.00 cm

Figure 10: This table shows the measurements of planaria seven days after the second measurements for the second trial

Spring Water	2-Apr	4-Apr	11-Apr
P1	0.70 cm	0.75 cm	0.85 cm
P2	0.90 cm	1.00 cm	1.15 cm
P3	0.60 cm	0.70 cm	0.75 cm
P4	0.75 cm	0.80 cm	0.90 cm
P5	0.80 cm	0.85 cm	1.00 cm
P6	0.75 cm	0.85 cm	0.95 cm

Figure 11: Three planaria were split into a total of six pieces and placed in spring water as the control. The table above shows the measurements taken over the nine days for the first trial

Spring Water	2-Apr	4-Apr	11-Apr
P1	0.50 cm	0.55 cm	0.65 cm
P2	0.90 cm	1.05 cm	1.15 cm
P3	0.60 cm	0.70 cm	0.85 cm
P4	0.70 cm	0.75 cm	0.90 cm
P5	0.75 cm	0.85 cm	0.90 cm
P6	0.60 cm	0.65 cm	0.75 cm

Figure 12: Three planaria were split in half and placed in spring water for the control group. The table above shows the measurements for trial two

Planaria in the creatine solution were still moving at an increased rate. The planaria were moving in a similar fashion to the observations from the first week. Planaria did seem a little less energetic, probably because they had not been fed in a while. It was unsure if the planaria were still going to be alive but were perfectly fine when checked.

b)   Results

Trial One
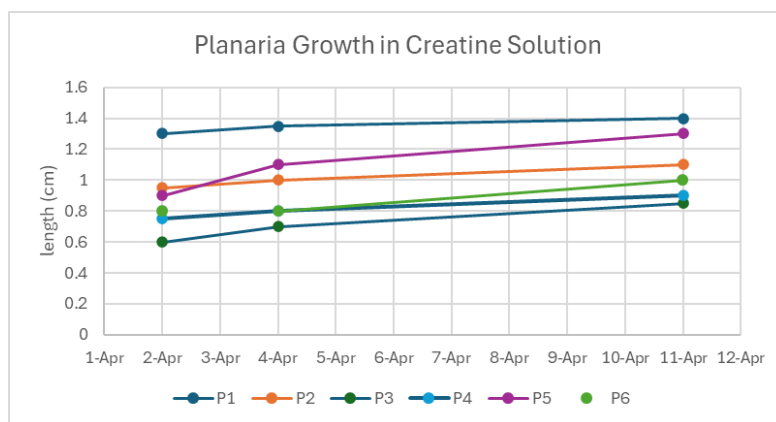
Figure 13: Planaria were measured two days after being split, then measured 7 days after the second measurement. This graph shows the growth of the planaria in the first trial. Each line represents the planaria pieces that were split in half
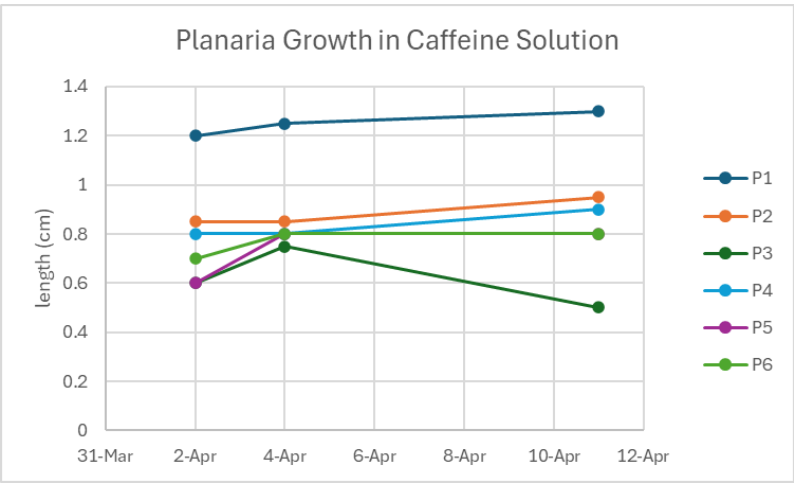
Figure 14: This graph shows the length of the caffeine solution planaria after they were split in half for the first trial. The lines represent the six planaria pieces
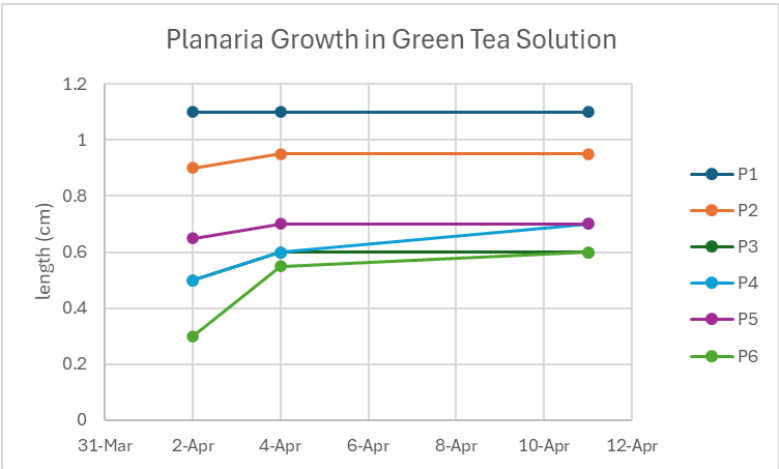
Figure 15: The lines represent the different planaria in the green tea solution. This graph shows the measurements of each piece after being split for trial one
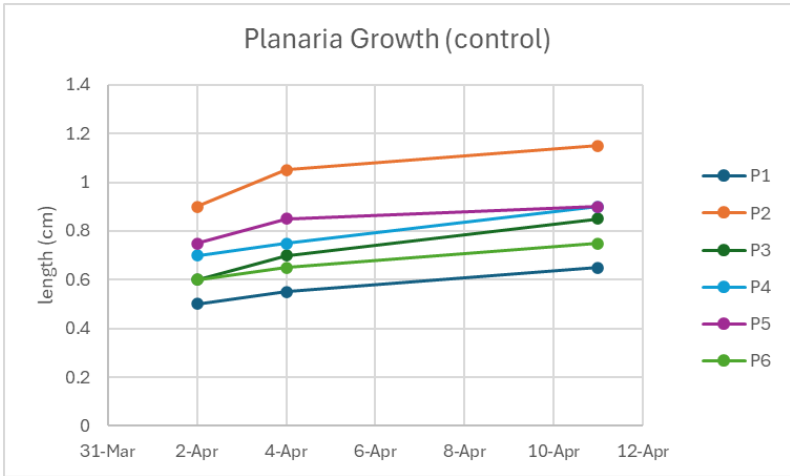
Figure 16: The graph shows the growth of the planaria in the control group over nine days. Each line represents a different planaria piece

	Creatine	Caffeine	Green Tea
P1	0.10 cm	0.10 cm	0 cm
P2	0.15 cm	0.10 cm	0.05 cm
P3	0.25 cm	-0.10 cm	0.10 cm
P4	0.15 cm	0.10 cm	0.20 cm
P5	0.40 cm	0.20 cm	0.05 cm
P6	0.20 cm	0.10 cm	0.30 cm
Average	0.208 cm	0.083 cm	0.117 cm

Figure 17: The total growth of each planaria in all the solutions was calculated. Using that data, the average growth was found for each variable group

	Spring Water
P1	0.15 cm
P2	0.25 cm
P3	0.25 cm
P4	0.20 cm
P5	0.15 cm
P6	0.15 cm
Average	0.192 cm

Figure 18: The table above shows the total growth each planaria in the control had. That data was used to calculate the average growth

Trial Two
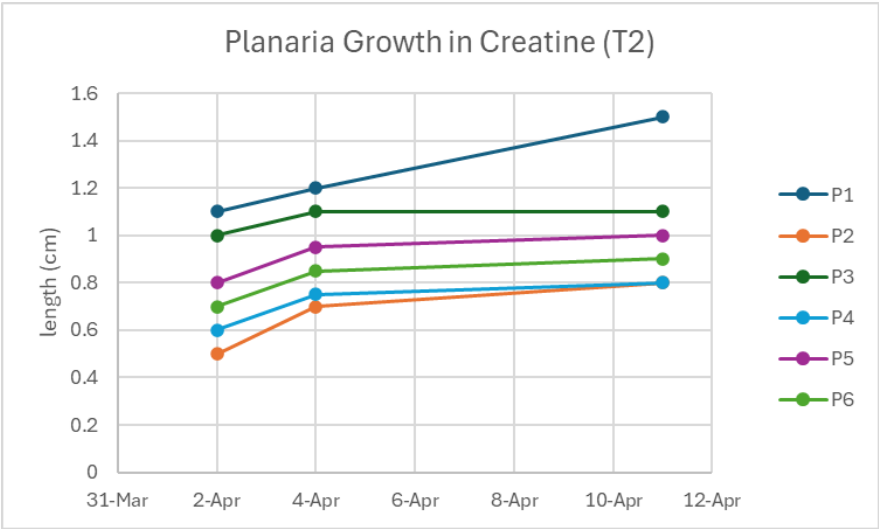
Figure 19: Planaria growth in the creatine solution was measured over nine days. The data in this graph was from trial two
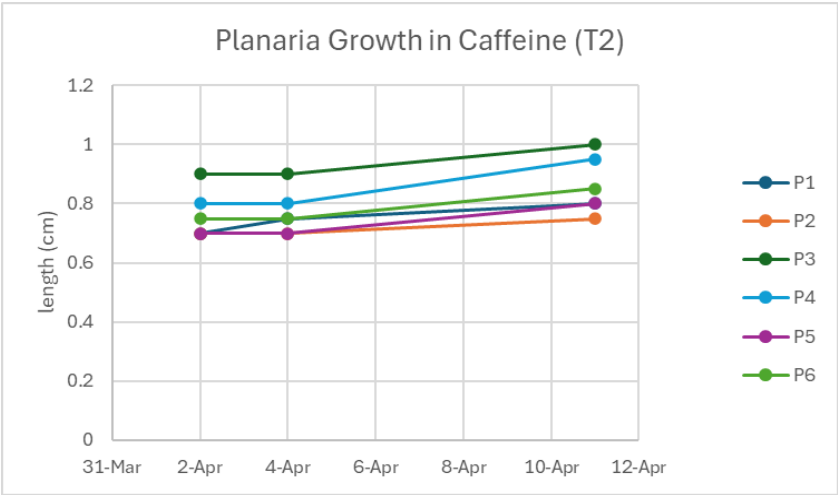
Figure 20: Planaria growth in the caffeine solution was measured over nine days. Each line represents the different planaria piece for trial two
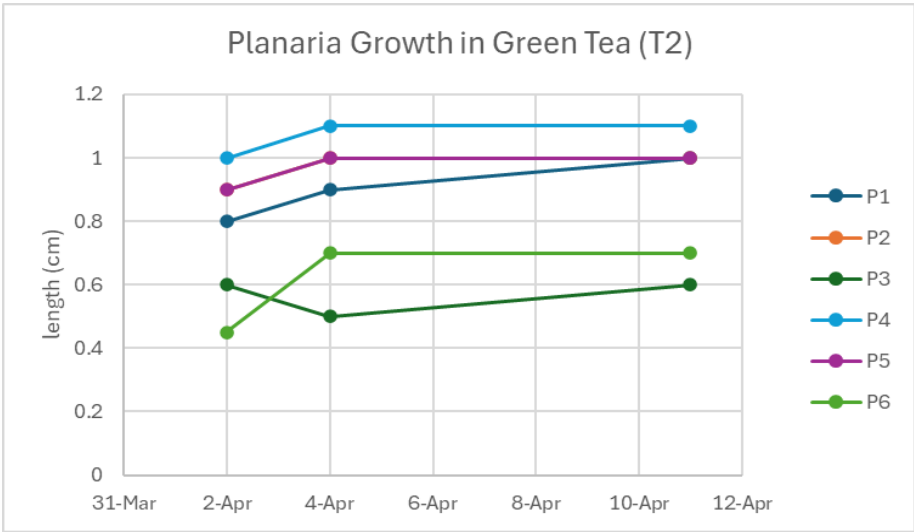
Figure 21: Planaria growth in green tea solution was measured over nine days. That graph above represents the planaria in trial two
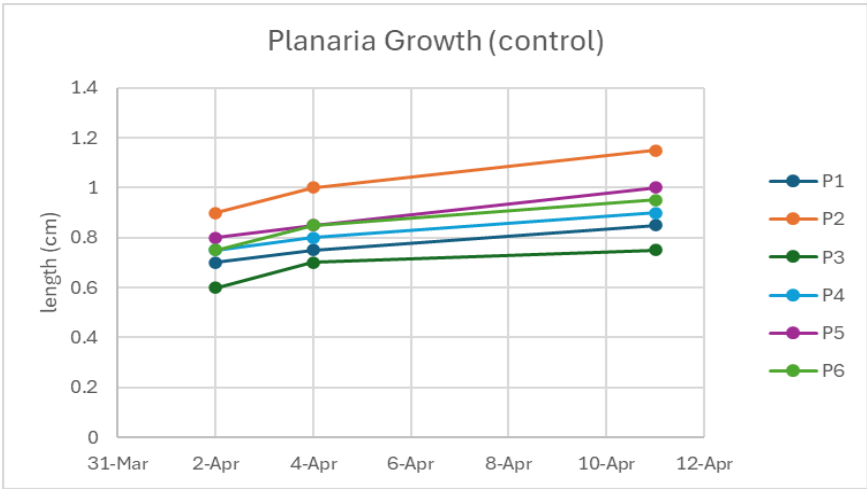
Figure 22: The graph shows the growth of the planaria in the control group over nine days. Each line represents a different planaria piece

	Creatine	Caffeine	Green Tea
P1	0.40 cm	0.10 cm	0.20 cm
P2	0.30 cm	0.05 cm	0.10 cm
P3	0.10 cm	0.10 cm	0.00 cm
P4	0.20 cm	0.15 cm	0.10 cm
P5	0.20 cm	0.10 cm	0.10 cm
P6	0.20 cm	0.10 cm	0.25 cm
Average	0.233 cm	0.10 cm	0.125 cm

Figure 23: The total growth of each planaria piece was calculated for trial two. Using this data, the average growth for each variable group was found:

	Spring Water
P1	0.15 cm
P2	0.25 cm
P3	0.15 cm
P4	0.15 cm
P5	0.20 cm
P6	0.20 cm
Average	0.183 cm

Figure 24: This table shows the total growth shown by the planaria in the control group. That data was used to calculate the average growth

IV. CHAPTER FOUR

a) Discussion

Analysis of the data from this experiment concluded that the creatine solution had the most regenerative capacity. This matches with what was hypothesized before the experiment. The researchers had hypothesized creatine to be more successful due to the extensive study that has been done on it to help show its benefits. Creatine had been known to be useful in supplying energy. Due to the difference in consumer, they had to adjust the concentrations of the creatine. They had conducted error trials to determine the lethal concentrations of the variables to the planaria. It showed them that 5% creatine solution and 15% green tea and caffeine solutions would be the limit of the planaria to test out on. After they completed their error trials, they noticed the many planaria remaining in beaker A had almost disappeared. Also, beaker B which was right next to it with a 5% concentration of creatine solution had gone from a light shade of pink to a foggy solution that appeared to have looked dirty. At first, they had thought that the planaria escaped into the beaker next to them. After analyzing the solution and talking with Dr. Romano, the biology professor at Governor's School, they had concluded that the planaria in the creatine solution had built up bacteria over the weekend and that

is what caused the foggy appearance in the beaker. The beaker with spring water and planaria had been left with chunks of the boiled egg in the water which had ended up choking the planaria and caused them to disintegrate into the water which is what gave the appearance of almost no planaria in there. After looking around the beaker and filtering and diluting some of the spring water. Only five of the planaria had been recovered out of the 24 that should have still been in the beaker.

In their caffeine solution, they had noticed the main increase in growth being in the beginning but even for typical planaria most growth tends to occur in the beginning stages of regeneration so the growth from the creatine is not significantly impressive. Caffeine had originally been selected for its ability to increase activity. It is a stimulant and can increase circulation. These traits appeared promising as planaria already possess regenerative potential, it could speed up its regeneration time. The caffeine had not met the expectations and showed negative effects on the planarian's regeneration time as the average growth of the planaria in spring water was still higher than with the caffeine solution. During the error trials, they had noticed the activity of the planaria in a concentration that ended up lethal be a lot more active than the planaria in the other solutions. After the set experiment concentrations, the planaria in the creatine solution became the most active. During one of

the data collection labs, the researchers noticed that the planaria in the caffeine solution had decreased as much as it has increased in the collection lab before.

In the green tea solution, they had noticed that there was a large increase in size in the beginning which was already expected but the increase had quickly decreased and became insignificant. As the experiment reached the final days, they noticed another significant increase in size in the end. The significance of the increase is in comparison to its previous data, and it compares to how the trajectory of the planarian growth was heading towards. The increase was unexpected but promising. They had not had enough time to complete another trial or lengthen the trials they were running but for future research it could be promising to look into. Green tea had been selected for naturally being known to have healing properties. The properties were being tested on the planaria and although it did take some time, it has the potential to have a significant effect on planarian regeneration. Unfortunately, its capacity to regenerate could not be fully studied. In place, the capacity of the creatine was promising and apparent. It quickly showed its effectiveness, especially in comparison to the constant trial. Overall, this experiment has been able to highlight creatine's healing features and possibilities. Through further research, it could be modified to have regeneration as an updated version's specific purpose.

b) Dedication

I dedicate this work to my friends Mahika, Tvisha, Rania, Alina, and Leul who have supported me throughout this year. Thank you for getting through chem together with me. I also thank Dr. Patanarut for teaching me and helping me learn. - Rosangela Le

I dedicate this work to Dr. P for teaching me for my first semester at the governor's school. Thank you for getting me through this tough transition from regular high school to college classes. - Leul Wondwossen

Acknowledgements

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Assessing Rubella Immunity: Seroprevalence among Pregnant Women in Brazzaville, Congo

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Marien Ngouabi University

Summary- Background: Rubella is a major public health problem because of its teratogenic effects, especially in early pregnancy. Understanding the serological profile of pregnant women is crucial to preventing congenital rubella syndrome. This study aimed to determine this profile in pregnant women affected by rubella.

Methodology: We conducted a prospective descriptive study on blood plasmas from pregnant women over a period of three months, from January 1 to March 31, 2022. A total of 98 blood plasma samples from pregnant women were analyzed. Sampling was carried out systematically on all blood samples from pregnant women who had attended the laboratory of the Blanche Gomez Mother-Child Specialty Hospital in Brazzaville, Congo, for biological investigations. IgG and IgM assay were performed using the Architect i1000SRanalyzer (ABBOTT), using the microparticle chemiluminescence immunological technique.

Keywords: rubella, pregnant women, seroprevalence, immunity.

GJMR-C Classification: QW 168.5.R8

Strictly as per the compliance and regulations of:

Assessing Rubella Immunity: Seroprevalence among Pregnant Women in Brazzaville, Congo

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Results: The overall IgG seropositivity of pregnant women was 91.8%. This rate was higher among women over 34 years of age, reaching 100%. The 16-24 and 25-34 age groups had 95.7% and 87.3% seropositivities, respectively. The distribution of the population by gestational age showed maximum seropositivity among women in the second trimester of pregnancy (97.5%), followed by those in the first trimester (88.4%) and third trimester (86.7%). Notably, 62.5% of pregnant women in the first trimester had a negative serology. Mean IgG titers were highest in women aged 25 to 34 years (94.3 IU/L) and in those in the third trimester of pregnancy (109.2 IU/L). IgG seroprevalence showed no statistically significant differences between age groups ($p = 0.405$) or between trimesters of pregnancy ($p = 0.376$). No pregnant women have been IgM positive.

Conclusion: IgG seropositivity (91.8%) shows strong immunity against rubella. No pregnant women developed IgM, indicating the absence of recent infections. Vulnerability in the first quarter (62.5%) remains a concern. Surveillance, awareness-raising and vaccination are essential to improve vaccination coverage and protect vulnerable populations in Congo, thereby reducing transmission and protecting future generations.

Keywords: rubella, pregnant women, seroprevalence, immunity.

Résumé- Contexte: La rubéole est un problème majeur de santé publique à cause de ses effets tératogènes, surtout en début de grossesse. Il est crucial de comprendre le profil sérologique des femmes enceintes pour prévenir le syndrome de rubéole congénitale. Cette étude visait à déterminer ce profil chez les femmes enceintes touchées par la rubéole.

Méthodologie: Nous avons mené une étude prospective descriptive sur des plasmas sanguins de femmes enceintes sur une période de trois mois, du 1er janvier au 31 mars 2022. Au total, 98 échantillons de plasmas sanguins de femmes enceintes ont été analysés. L'échantillonnage a été effectué de manière systématique sur l'ensemble des prélèvements sanguins des femmes enceintes ayant fréquenté le laboratoire de l'hôpital spécialisé mère-enfant Blanche Gomez à Brazzaville, Congo, pour des investigations biologiques. Le dosage des IgG et IgM a été réalisé à l'aide de l'analyseur Architect i1000SR (ABBOTT), utilisant la technique immunologique microparticulaire par chimiluminescence.

Résultats: La séropositivité globale en IgG des femmes enceintes était de 91,8 %. Ce taux était plus élevé chez les femmes de plus de 34 ans, atteignant 100 %. Les groupes d'âge de 16 à 24 ans et de 25 à 34 ans avait respectivement des séropositivités de 95,7% de 87,3 %. La répartition de la population selon l'âge gestationnel a montré une séropositivité maximale chez les femmes au deuxième trimestre de grossesse (97,5 %), suivie de celles au premier trimestre (88,4 %) et au troisième trimestre (86,7 %). Notamment, 62,5 % des femmes enceintes au premier trimestre avaient une sérologie négative. Les titres moyens d'IgG étaient les plus élevés chez les femmes âgées de 25 à 34 ans (94,3 UI/L) et chez celles au troisième trimestre de grossesse (109,2 UI/L). La séroprévalence des IgG n'a montré aucune différence statistiquement significative entre les groupes d'âge (p = 0,405) ni entre les trimestres de grossesse (p = 0,376). Aucune femme enceinte n'a été séropositive aux IgM.

Conclusion: La séropositivité en IgG (91,8 %) montre une forte immunité contre la rubéole. Aucune femme enceinte n'a développé d'IgM, indiquant l'absence d'infections récentes. La vulnérabilité au premier trimestre (62,5 %) reste préoccupante. Surveillance, sensibilisation et vaccination sont essentielles pour améliorer la couverture vaccinale et protéger les populations vulnérables au Congo, réduisant ainsi la transmission et protégeant les futures générations.

Mots-clés: rubéole, femmes enceintes, séroprévalence, immunité.

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I. Introduction

Rubella is an acute illness caused by the rubella virus, manifesting as a maculopapular rash and fever. Highly contagious, it is transmitted through the air through direct contact with an infected person, whose nasopharyngeal secretions contain the virus (1, 2). Generally benign and mainly affecting children, rubella is however a major public health problem due to its teratogenic potential, especially in early pregnancy. Infection during the first trimester can lead to miscarriages, fetal deaths, stillbirths, or birth defects (up to 90% of cases), known as congenital rubella syndrome (CRS). This syndrome can affect various organ systems, including the ophthalmic, auditory, cardiac, neurological, hepatic, and hematological systems (3). Understanding the serological profile is essential to protect the health of mothers and babies, by enabling a timely and effective intervention. The WHO estimates that each year, about 100,000 cases of CRS occur worldwide, including 39,000 in Africa in 2010. The risk of CRS is highest in countries with high rates of rubella susceptibility in women of childbearing age. The incidence of CRS has been significantly reduced or eliminated in many regions due to effective vaccination programs (4, 5). However, rubella remains endemic in several resource-limited countries, particularly in sub-Saharan Africa (6, 7).

The rubella vaccine is an effective prophylactic measure to control the spread of the virus and CRS. However, the devastating consequences of infection persist, not least due to the presence of unprotected people, such as those with ethical or religious objections to vaccination or those who have migrated from areas without adequate vaccination coverage (8, 5). In Congo, the Expanded Program on Immunization (EPI) introduced the combined measles and rubella vaccine in 2019. This first national campaign aimed to reduce morbidity and mortality due to measles and rubella among children, reaching a vaccination coverage rate of 96.9%, although disparities exist between departments (9). Children with CRS can suffer from hearing loss, eye and heart defects, and other lifelong conditions (including autism, diabetes mellitus, and thyroid dysfunction), often requiring expensive treatments and surgeries. The risk of CRS is highest in countries where women of childbearing age are not immune. The seroprevalence of rubella in pregnant women has been studied in several African countries (10-15). A meta-analysis reported a seroprevalence of 89.0% among pregnant women in sub-Saharan Africa (16). In Central Africa, high seroprevalence has been reported, including in Gabon (87.56 per cent) (17), the Democratic Republic of the Congo (84 per cent) (18) and Cameroon (94.4 per cent) (19). Also in Cameroon, another study reported 10.2% of probable cases of CRS (20). In Congo, the epidemiology of rubella remains insufficiently documented, but WHO estimates that Central Africa has high incidences, and Congo is no exception. In 1991, Yala et al. reported an 85% seroprevalence among pregnant women (21). Understanding the serological profile of pregnant women is crucial to preventing CRS. The objective of this study was to determine the serological profile of women pregnant with rubella in order to improve prevention and public health protection strategies.

II. Materials and Methods

a) Ethical Considerations

This study was carried out using anonymized blood samples, in accordance with current ethical regulations. As the samples are completely anonymous and cannot be linked to any personally identifiable data, an authorization from the ethics committee was not required.

b) Type, Period and Setting of the Study

This was a prospective descriptive study to determine the serologic profile of pregnant women with rubella. Such a study provides a valuable basis for future research in public and medical health.

This study involved 98 anonymous blood plasma samples from pregnant women. They attended the laboratory of the Blanche Gomez Mother-Child Specialty Hospital in Brazzaville, Congo, for biological investigations between January 1 and March 31, 2022. The laboratory analysis was designed to detect the presence of IgG and IgM antibodies against the rubella virus. The plasma samples, stored at -20°C, were transferred and stored at the National Public Health Laboratory in Brazzaville. They were then transported by air to the Institute Pasteur de Côte d'Ivoire in Abidjan for analysis. The samples were carefully packaged in triple packaging in insulated bags equipped with cold packs, to ensure their integrity. Maintaining the integrity of plasma samples is essential to obtain reliable and accurate results when testing rubella antibodies. This precaution is essential to obtain reliable and accurate results when testing rubella antibodies.

c) Sampling

Systematic sampling was based on all venous blood samples from pregnant women during the study period, collected in tubes containing an anticoagulant (EDTA). Variables analyzed included the age of pregnant women and gestational age. Clinical (gestational age) and epidemiological (age) data were collected using survey sheets including: a code assigned to the sample for the study, the age, and the gestational age.

d) Methodology

i. Collection of blood plasma samples

After the laboratory tests requested by the patients, the plasma was separated from the whole blood. The venous blood samples, collected in tubes

containing an anticoagulant (EDTA), were centrifuged at 3000 revolutions per minute for 5 minutes. The obtained plasmas were aliquoted into 1.5 ml Eppendorf tubes and stored at -20°C

ii. Dosing principle

Rubella antibody titers were determined using the Architect *i1000SR* analyzer, using chemiluminescent microparticle immunoassay technology. This system relies on paramagnetic microparticles as a solid phase for the quantitative and qualitative detection of rubella antibodies in serum samples. The chemiluminescence signal is measured in relative units of light (RLU), which are directly proportional to the concentrations of immunoglobulins in the serum samples.

The higher the antibody concentrations, the greater the number of photons detected.

iii. Sample Analysis

For assays, the samples were sent to the Bacterial and Viral Serology Unit (USBV) of the Institute Pasteur de Côte d'Ivoire. Plasma samples were allowed to thaw at room temperature. Prior to the analysis, the parameters of interest (IgG and IgM) were calibrated on our samples. This calibration, which is stable for several months, must be verified with at least two levels of control. Once the plasmas were thawed, 200 μL of plasma was transferred to the cups of the Architect *i1000SR* analyzer for scheduled sample analysis. After analysis, the results were printed and the samples refrozen at -20°C for possible reuse. Quality control was ensured by introducing two levels of control of known concentrations in each series of analyses, ensuring the precision and accuracy of the analytical system and detecting random (pipetting, mixture quality, cup cleanliness, photometric instability) and systematic (loss of calibration) errors.

iv. Interpretation

For IgM, a result was considered positive (reactive) when the sample index was ≥ 1.60, negative (non-reactive) when the index was < 1.20, and equivocal when the index was between 1.20 and 1.59. For IgG, a positive result was considered when the IgG titer was ≥ 10.0 IU/mL, negative between 0 and 4.9 IU/mL, and equivocal between 5.0 and 9.9 IU/mL. Subjects with IgG titers ≥ 10.0 IU/mL were considered immune; those with titers < 10.0 IU/mL, as non-immunized. In this study, equivocal results were considered negative.

e) Data Analysis

Data was collected and analyzed using Microsoft Office Excel 2019. The Fisher exact test was used to assess the relationship between seropositivity and epidemiological and clinical characteristics, with a statistical significance level of 5%.

III. RESULTS

a) Epidemiological and Clinical Data

The study population consisted of 98 blood plasma samples from pregnant women. The distribution by age group made it possible to distinguish three age groups. The mean and median age were 29.04 years and 29 years, respectively. The ages of pregnant women ranged from 16 to 43 years. The most represented age group was 25 to 34 years old (56.1%). The majority of pregnant women were in the first trimester of pregnancy (43.9%) (Table I).

Table I: Distribution of Pregnant Women by Age and Trimester of Pregnancy

Variables	Effective (n=98)	%
Age group (year)		
16 - 24	23	23,5
25 – 34	55	56,1
≥ 34	20	20,4
Gestational age (Quarter)		
1st	43	43,9
2nd	40	40,8
3rd	15	15,3

b) Epidemiological and Clinical Data by IgG Seropositivity

The overall IgG seropositivity rate was 91.8%. This rate was highest among women over 34 years old (100%), followed by the age groups 16 to 24 years (95.7%) and 25 to 34 years (87.3%). The distribution of the population by gestational age showed a maximal seropositivity among women in the second trimester of pregnancy (97.5%), followed by those in the first trimester (88.4%) and in the third trimester (86.7%). Additionally, 8.2% of women had a negative IgG result, with 62.5% of these women being in the first trimester of pregnancy. No pregnant woman tested positive for IgM. Seropositivity showed no statistically significant association with age (p = 0.405) or trimester of pregnancy (p = 0.376) (Table II).

Table II: Distribution of Epidemiological and Clinical Data by IGG Seropositivity

Variables	No. of samples	IgG+ (n=90)		IgG- (n=8)	Titter IgG (UI/L)
		n (%)	P value	n (%)	mean (SD)
Age group (year)					
16–24	23	22 (95,7)		1 (4,3)	83,2 (69,9)
25-34	55	48 (87,3)	0,405	7 (12,7)	94,3 (104,4)
≥ 35	20	20 (100)		0 (0)	82,8 (61)
Gestational age (Quarter)					
1st	43	38 (88,4)		5 (11,6)	89,7 (83)
2nd	40	39 (97,5)	0,376	1 (2,5)	81,46 (68,8)
3rd	15	13 (86,7)		2 (13,3)	109,2 (143,3)

Table III: Seroprevalence of rubella among pregnant women in selected African countries

Authors, year of publication	Country	Study area	Study population	Sample size	Rubella (%) IgG+	IgM+	Dosing technology
Taku et *al.* 2019	Cameroon	Urban	Pregnant women	522	94,4	5,0	ELISA
Pegha Moukandja et *al.* 2017	Gabon	Urban	Pregnant women	973	87,56		ELFA
Alleman et *al.* 2016	Ground floor	Urban/Rural	Pregnant women	1605	84		ELISA
Zahir et *al.* 2020	Morocco	Urban area	Pregnant women	380	84,7	0	CMIA
AlShamlan et *al.* 2021	Saudi Arabia	Urban	Pregnant women	4328	76,41	1,21	CLIA
Tahita et *al.* 2013	Burkina Faso	Urban/Rural	Pregnant women	341	95		ELISA
Adam et *al.* 2013	Sudan	Urban	Pregnant women	500	95,1		ELISA
Adewumi et *al.* 2015	Nigeria	Urban	Pregnant women	272	91,54	1,84	ELISA

CMIA: Chemiluminescence Microparticle Immunoassay
CLIA: Chimiluminescence Immunoassay
ELISA: Enzyme-Linked Immunosorbent Assay

IV. Discussion

The elimination of congenital rubella and the prevention of congenital rubella syndrome (CRS) are major global public health issues. The World Health Organization (WHO) estimates that about 100,000 cases of CRS occur worldwide each year. In 2010, there were an estimated 39,000 cases of CRS in Africa (5). These alarming figures underscore the critical need for routine rubella screening in pregnant women and widespread rubella vaccination in the population (22).

The ages of the pregnant women studied ranged from 16 to 43 years, with an average age of 29 years. The 25 to 34 age group was the most represented (56.1%) (Table I). Zahir et *al*. in Morocco (23) also reported, in agreement with this study, a predominance in the 25-34 age group (50.8%) among pregnant women. Our average age was higher than the averages reported by Taku et *al*. (27 years old) in Cameroon (19) and Pegha Moukandja et *al*. (25 years old) in Gabon (17).

In this study, the majority of pregnant women were in their first trimester of pregnancy (43.9%). Trends vary between studies: Taku et *al*. reported a higher frequency of women in the second trimester of pregnancy (59.6%) (19), while AlShamlan et *al*. observed a majority of cases in the first trimester (38.89%) in Saudi Arabia (24). Taku et *al*. in Cameroon and Ekuma et *al*. in Nigeria reported a majority frequency (41% and 45.9%, respectively) in the third trimester of pregnancy (19, 25).

The epidemiology of rubella remains poorly known in Congo, as it is not a notifiable disease. Most acute infections are acquired in childhood and continue to manifest as IgM antibodies, even in adulthood. The results of this study revealed a high prevalence of IgG seropositivity among pregnant women who attended the Blanche Gomez Mother-Child Specialty Hospital. With an overall seroprevalence rate of 91.8%, it appears that the majority of pregnant women are protected against rubella. However, the detailed analysis shows disparities according to age and trimester of pregnancy.

The highest seroprevalence was observed in women over 34 years of age, with a rate of 100%. This finding suggests that older women have been exposed to the rubella virus during their lifetime, which has led to the development of antibodies and lifelong immunity. In contrast, some women under the age of 35 were unprotected, which could be attributed to insufficient exposure to the virus or incomplete vaccination.

The results also indicate that seroprevalence is higher during the first trimester of pregnancy, reaching 97.5%. This observation is crucial, as rubella virus infection in the first trimester can have serious consequences on fetal development, including congenital rubella syndrome.

The national measles and rubella vaccination programme for young children, launched in March 2019 (9), is an important step towards rubella elimination. However, the risk of infection in women of childbearing age does not decrease immediately, as they were not vaccinated as children. The lack of a routine immunization program prior to 2019 means that older women likely acquired immunity through natural infection, creating heterogeneity in the population. With the introduction of the new vaccination program, we anticipate an increase in immunity levels in future cohorts of pregnant women. This program is expected to homogenize protection against rubella and reduce the risk of CRS in the long term. By monitoring the effectiveness of the program, strategies can be adjusted to ensure optimal immunization coverage and improve public health.

Before 2019, the rubella vaccine (Aventis-Pasteur measles, mumps and rubella vaccine) was only available in a few private pharmacies and rarely used. In the absence of a mass vaccination campaign prior to 2019, and based on our clinical information indicating a very low vaccination rate, we conclude that the seroprevalence observed in this study is mainly due to the circulation of wild-type rubella virus rather than vaccination. These data suggest significant previous exposure to the virus and likely significant transmission in the city. Previous studies have shown that the rubella virus is common in several countries in sub-Saharan Africa (16, 26). Our seroprevalence rate was lower than that reported among pregnant women in Cameroon (94.4%) (19), Burkina Faso (95%) (27) and Sudan (95.1%) (28), but similar to that observed in Nigeria (91.5%) (29). In contrast, our prevalence was higher than in Gabon (87.56%) (17) and the Democratic Republic of the Congo (84%) (18). These differences could be due to sample sizes, disease endemicity, diagnostic methods, or test cut-offs.

The results of the statistical analysis, using the exact 5% Fisher test, show that seropositivity has no statistically significant association with either age (p = 0.405) or trimester of pregnancy (p = 0.376). These results suggest several important points to consider in interpreting the data and the implications for public health. The lack of a statistically significant association between seropositivity and the variables age and trimester of pregnancy indicates that other factors may play a more significant role in the presence of rubella antibodies in pregnant women. It is possible that factors such as vaccination history, individual medical history, or environmental exposure may be more influential in the observed seroprevalence.

This study found that 8.2% of pregnant women were not protected against rubella. In addition, 62.5% of non-immunized women were in the first trimester of pregnancy. Our results also showed that all women are at increased risk of rubella infection over the course of their lives. Since up to 90% of rubella infections occurring just before conception and up to the first 8–10 weeks of pregnancy can lead to multiple birth defects, miscarriage or stillbirth (30). These data indicate that a significant proportion of pregnant women are at risk of having a child with congenital rubella syndrome (CRS). The main goal of rubella vaccination programs is to prevent CRS by avoiding infections during pregnancy. To achieve this goal, all women of childbearing age must be vaccinated and vaccination coverage must be achieved above 95% among children. In some developed countries, pregnant women are routinely screened to offer postpartum vaccination to susceptible women (31). WHO recommends that all pregnant women who are HIV-negative or whose immune status is unknown should be vaccinated after delivery before leaving the hospital, in order to achieve 100% seroprevalence (32). In Congo, vaccination of postpartum women is not systematic and vaccination of women of childbearing age is not part of the vaccination programme. Reducing the risk of CRS will only be possible if the circulation of the virus is interrupted by

mass vaccination of women of childbearing age and school-age girls, routine vaccination of nonimmunized women after childbirth, vaccination of children against measles and rubella, as well as the establishment of a national surveillance system for rubella infection during pregnancy.

Specific IgM can be detected not only in cases of recent primary infection, but also in cases of reinfection, non-specific polyclonal stimulations of the immune system, or cross-reactions with rheumatoid factors in systemic disease (33). During this study, no pregnant women were IgM positive and there were no acute infections. Zahir et *al.* (23) also reported a positivity rate of 0%, in line with our study, while low rates were recorded among pregnant women in Cameroon (5%) (19) and Nigeria (1.84%) (29).

The lack of knowledge about the epidemiology of rubella in Congo is crucial. It would be appropriate to launch specific epidemiological studies and to set up continuous surveillance programmes. These initiatives would provide a better understanding of the dynamics of rubella transmission in the region and strengthen efforts to prevent and control the disease.

Limitations of the study: It is essential to consider the limitations of this study. First, the small size of our sample, although relevant to the study of pregnant women in Congo, may influence the generalization of the results. Second, the lack of a confirmed history of rubella vaccination precludes an assessment of the impact of vaccination on HIV status. In addition, unmeasured variables, such as socioeconomic status or antenatal care practices, could affect the findings. Finally, the results concern only women who attended the Blanche Gomez Mother-Child Hospital, thus limiting the scope of the conclusions. Despite these limitations, the study highlights the need for further research and effective prevention strategies to protect pregnant women and their children.

V. Conclusion

No pregnant women developed IgM, indicating the absence of recent or active infections. IgG seropositivity was high (91.8%), indicating strong immunity to rubella in these women. These findings highlight the importance of ongoing surveillance and vaccination for long-term protection. The vulnerability of women in the first trimester of pregnancy (62.5%) is of concern due to risks to fetal development and the high risk of congenital rubella syndrome. Awareness campaigns, partnerships with health care providers and continued immunization efforts are key to improving immunization coverage and protecting vulnerable populations. Achieving high levels of immunity in women of reproductive age is crucial for public health, as it can reduce rubella transmission and improve overall health in Congo, while preventing future outbreaks and protecting future generations. Monitoring the impact of vaccination campaigns and assessing the epidemiology of rubella is essential to adjust public health strategies and ensure continued protection.

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Serological Syphilis Testing in Brazilian Older Adults

By LaisKimie Tomiura, Fernanda Gehlen, Gustavo Henrique Morcelli Costa,
Sonia Maria Raboni & Renato Nisihara

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Abstract- Objectives: Syphilis is a silent disease detectable via serological tests, though interpreting results can be complex, particularly in older adults. This study examined laboratory findings in older adults who tested positive for syphilis through serological tests.

Methods: A retrospective study was conducted in Curitiba, Brazil, involving individuals over 60 years old, of both sexes, who tested positive for CMIA syphilis tests but negative for VDRL. FTA-ABS was performed as a confirmatory test, and demographic/clinical data were collected from medical records.

Results: A total of 311 patients were studied, 53.1% male, with a median age of 69 years. No significant differences were found between sexes or ages. Previous syphilis diagnoses were found in 7.4% of patients, and 10.2% had undergone prior treatment for syphilis. Of the patients, 23.4% came from internal medicine, 18.3% from neurology, and 17.3% from infectious disease clinics. False-positive CMIA results were observed in 14.1% of cases.

Keywords: syphilis, serologic test, aging adults.

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Conclusion: No significant differences were found between sexes or ages in patients with positive serologic syphilis tests. False-positive CMIA results were observed in 14.1% of cases, most of which had borderline values.

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I. Introduction

Syphilis is a systemic, chronic, and curable bacterial infection that, when untreated, can progress to severe stages, affecting various body systems, including the cardiovascular and nervous systems. [1] In Brazil, the detection rate of syphilis rose by 32.9% in 2021, particularly in patients over 60, increasing from 59.1 to 78.5 cases per 100,000 inhabitants. [2,3] Between June 2011 and June 2022, over 1.1 million cases of acquired syphilis were reported, with the majority being male (60.6%) and within the age groups of 20-29 (35.6%) and 30-39 (22.3%). [2]

To diagnose syphilis, two types of serological tests are used: non-treponemal tests (e.g., VDRL) and treponemal tests (e.g., CMIA and FTA-ABS). Treponemal tests, which are more specific, have been recommended as the first diagnostic step in Brazil since 2011. [4,5] A positive result in both non-treponemal and treponemal tests indicates active syphilis. [6] However, an isolated positive treponemal test may signal a past infection or a false-positive result. [5]

Routine syphilis testing in older adults is often performed to investigate conditions such as dementia or cardiovascular diseases. [7] However, diagnosing syphilis, especially in its late latent stage or in cases of neurosyphilis, poses clinical challenges. [8] VDRL positivity tends to decline over time, even without treatment, particularly in tertiary syphilis. [4]

Despite these challenges, few studies have focused on syphilis in older adults. In clinical practice, serological tests are the primary diagnostic tool, but limitations in non-treponemal test specificity and weak correlations between treponemal test results and disease activity complicate diagnoses. [8] Therefore, this study aimed to assess the epidemiological profile and laboratory findings in older adults with positive serological tests for syphilis and to evaluate the frequency of false-positive CMIA results.

II. Methods

a) Study Design and Ethical Considerations

This cross-sectional study, utilizing retrospective data collection, was approved by the local Research Ethics Committee (protocol number 4.754.505). The study adhered to the Good Clinical Practice guidelines and the ethical principles outlined in the Declaration of Helsinki.

b) Participants

This study utilized a convenience sample of patients aged 60 and older, of both sexes, drawn from the database of the Clinical Laboratory at the Complexo Hospital de Clínicas of the Federal University of Paraná (CHC-UFPR) between March 2019 and October 2022. Included were cases with divergent results, specifically positive treponemal (CMIA) and negative non-treponemal (VDRL) tests for syphilis. A third treponemal test (FTA-ABS) was performed to confirm the serological results. Additional demographic and clinical data, including sex, age, previous syphilis diagnosis, and prior treatment history, were extracted from medical records.

c) Serological Testing For Syphilis

All serological tests were conducted at the Clinical Analysis Unit Laboratory of Hospital de Clínicas, Curitiba, Brazil. The precision of the quantitative tests

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was evaluated following Clinical and Laboratory Standards Institute (CLSI) guidelines (EP15-A3). [9] Three levels of quality control materials were used. Treponemal tests were performed using CMIA (Alinity System, Abbott, USA), with a manufacturer-defined cutoff point of 1.0. Patients with positive CMIA results underwent a non-treponemal VDRL test (Wierner, Argentina) at dilutions of 1:1 and 1:10 to avoid the prozone effect. Additionally, FTA-ABS tests (Wama Diagnostic, Brazil) were performed with an initial dilution of 1:4, and samples were treated with an absorbent buffer for 30 minutes. The indirect immunofluorescence reaction was observed under a microscope (Olympus, Japan) by a single examiner. As per Brazilian Ministry of Health guidelines, a false-positive syphilis result is determined when a positive CMIA is not confirmed by FTA-ABS. All non-concordant cases were retested for verification.

d) Statistical Analysis

Data were collected in frequency and contingency tables. Nominal and categorical data are expressed as percentages. The distribution of numerical data was performed using the Shapiro–Wilk test and central tendency expressed as means and standard deviations for parametric data and median and interquartile range (IQR) for non-parametric data.

The chi-squared test was used to compare nominal data, while the unpaired t-test and Mann-Whitney test were used to compare numerical data. The adopted significance level was 5%.

III. Results

A total of 311 patients with positive CMIA anti-treponemal tests were included in the study, comprising 146 females (46.9%) and 165 males (53.1%; $p = 0.12$), with a median age of 69 years (IQR = 64-75). No significant differences were found when comparing age and sex ($p = 0.35$). Of the patients, 23 (7.4%) reported prior syphilis diagnosis, and 30 (10.2%) had received treatment. Among those treated, 63.3% were male (19/30) and 36.6% were female (11/30).

Tests were performed on patients from various departments: 73 (23.4%) from internal medicine, 57 (18.3%) from neurology, and 54 (17.3%) from infectious diseases, all with a median age of 69 years. Of the 54 infectious diseases patients, 28 (12.3%) were HIV follow-up patients, of which 16 were male (57.2%) and 12 female (42.8%). Across the hospital, 14 medical specialties requested syphilis tests.

As shown in *Figure 1,* in 44 patients (14.1%), there was a discordance between treponemal test results: positive CMIA and negative FTA-ABS, indicating a false-positive result as per Brazilian guidelines (2022). [2] Among the group with both reactive treponemal tests ($n = 267$), 58% were male, while the discordant group had 40.1% males and 59.9% females ($p = 0.07$).

The median age in the false-positive CMIA group was 67 years (IQR = 65-74), while the group with both positive tests had a median age of 69 years (IQR = 64-75), showing no significant difference ($p = 0.86$). Of the 311 patients, 46 (14.8%) had CMIA values between 1.1 and 2.0, considered positive. In this subgroup, 24 (52.1%) tested negative on FTA-ABS, 13 (28.2%) tested mildly positive, and 9 (19.5%) were weakly positive, leading to diagnostic uncertainty in over half of these cases.

Chemiluminescence values *(Figure 2)* showed a median optical density (OD) of 4.9 (IQR = 2.6-8.8) in the true-positive group, while the false-positive group had significantly lower OD values (median 1.9; IQR = 1.2-2.4; $p < 0.0001$). However, it is noteworthy that some cases with low CMIA values also yielded positive FTA-ABS results.

IV. Discussion

The findings from this study contribute to a deeper understanding and expand discussions on interpreting syphilis serological tests in older adults. The increased likelihood of false-negative results in non-treponemal tests, especially in this population, raises concerns about distinguishing between a serological scar and late latent syphilis. In the late latent phase, the sensitivity of the VDRL test ranges from 34-94% in the general population, and even without treatment, non-treponemal test titers may revert to negative. [1] This makes accurate diagnosis challenging, particularly in older individuals.

Since 2013, Brazil has experienced a marked rise in syphilis cases, particularly among older adults. This trend underscores the need to include syphilis in the differential diagnosis of other systemic diseases in this population and to raise awareness about transmission and prevention. According to the Brazilian Institute of Geography and Statistics (IBGE), by 2050, older adults will represent 30% of Brazil's population.[7] This demographic shift, coupled with increased longevity, improved quality of life, and extended sexual activity, emphasizes the importance of addressing syphilis in older adults. Notably, studies suggest individuals aged 50-64 are least likely to use condoms globally. [10, 11].

In our sample, no significant differences were found in the positivity of syphilis serological tests between males and females. However, Ministry of Health data indicate that men over 50 are more likely to be diagnosed with syphilis than women.[2] This disparity may be related to the setting of the study, which was conducted in a tertiary hospital, potentially reflecting a higher male representation in more complex or advanced cases referred for specialized care.

In terms of prior syphilis diagnosis awareness, only 7.4% of patients reported a previous diagnosis in

their medical records. Bastos et al. found that around 68% of older adults were unaware of syphilis, and 70.9% did not know how the disease is transmitted. [12] Additionally, healthcare providers often feel uncomfortable discussing STIs with older patients, which can contribute to underreporting and insufficient communication regarding syphilis and its transmission in this age group.

Internal medicine, neurology, and infectious diseases were the specialties that most frequently requested syphilis testing in this study. In contrast, specialties that commonly order tests in younger adults, such as gynecology, urology, and dermatology, were less represented in the sample. The high number of requests from neurology can be attributed to the protocol recommendation for syphilis testing in patients with dementia or suspected neurosyphilis. [13] Treponemal antibody tests typically remain positive long-term, whether the infection is treated or not, while non-treponemal tests may become negative in late neurosyphilis. [14] In cases where both treponemal tests (CMIA and FTA-ABS) are positive but VDRL is negative, a lumbar puncture is advised for confirmation. It's important to note that while VDRL is the recommended test for cerebrospinal fluid, it has low sensitivity (about 53%) but high specificity. [15]

In the studied sample, following Brazilian guidelines, 14.1% of CMIA tests were negative on the FTA-ABS, indicating false-positive results. This is likely due to the high sensitivity of CMIA, which may detect non-specific anti-treponemal antibodies during FTA testing. [16] False-positive reactions occur in about 1% of the general population in treponemal tests. [17] The age of the sample may contribute to the presence of non-specific antibodies, as previous studies have shown that the risk of false positives increases with age. [18] Additionally, CMIA OD values were significantly higher in cases where both treponemal tests were positive. It is important to note that many false-positive cases had CMIA values close to the cutoff threshold. Transient false-positive results can also arise due to infections, vaccinations, medications, blood transfusions, or pregnancy. [17] However, limited research on syphilis serological tests in older adults makes comparisons with other studies challenging.

This study has certain limitations due to its retrospective design. The lack of access to full medical records prevented us from gathering detailed information about the final diagnoses and whether syphilis treatment was administered. Despite these limitations, the findings underscore the need for the development of new and more reliable diagnostic tests for syphilis, particularly in older adults, where interpretation can be challenging.

Our study also contributes to the growing body of knowledge regarding the interpretation of syphilis serological tests in older populations. The higher likelihood of false-negative results in non-treponemal tests in this age group raises concerns about accurately distinguishing between a serological scar and late latent syphilis. As a result, careful interpretation is crucial to avoid misdiagnosis and ensure appropriate treatment decisions.

V. CONCLUSION

This study found that the median age of older patients with positive serologic tests for syphilis was 69 years, with no significant differences between sexes. The medical specialties with the highest number of positive syphilis tests were internal medicine, neurology, and infectious diseases. False-positive results were observed in 14.1% of CMIA cases, with most having borderline values. There were no significant associations between age or sex and the occurrence of false-positive serologic tests for syphilis in this older population.

Conflict of Interest Statement
All authors declare that we have no potential conflict of interest.

Ethics Approval: All procedures performed in studies involving human participants were in accordance with the institutional research ethics committee standards and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study was approved by the Research Ethics Committee from the Evangelic Mackenzie School of Medicine under protocol number 4.754.505.

Transparency Declaration
The authors affirm that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

Abbreviations:
CMIA= Chemiluminescent microparticle immunoassay (treponemic test)
FTA-ABS= Fluorescent treponem antibody absorption (treponemic test)
VDRL= Veneral Disease research in the laboratory (non-treponemic test)

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- Abstract: font size 9 with the word "Abstract" in bold italics.
- Main text: font size 10 with two justified columns.
- Two columns with equal column width of 3.38 and spacing of 0.2.
- First character must be three lines drop-capped.
- The paragraph before spacing of 1 pt and after of 0 pt.
- Line spacing of 1 pt.
- Large images must be in one column.
- The names of first main headings (Heading 1) must be in Roman font, capital letters, and font size of 10.
- The names of second main headings (Heading 2) must not include numbers and must be in italics with a font size of 10.

Structure and Format of Manuscript

The recommended size of an original research paper is under 15,000 words and review papers under 7,000 words. Research articles should be less than 10,000 words. Research papers are usually longer than review papers. Review papers are reports of significant research (typically less than 7,000 words, including tables, figures, and references)

A research paper must include:

a) A title which should be relevant to the theme of the paper.
b) A summary, known as an abstract (less than 150 words), containing the major results and conclusions.
c) Up to 10 keywords that precisely identify the paper's subject, purpose, and focus.
d) An introduction, giving fundamental background objectives.
e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition, sources of information must be given, and numerical methods must be specified by reference.
f) Results which should be presented concisely by well-designed tables and figures.
g) Suitable statistical data should also be given.
h) All data must have been gathered with attention to numerical detail in the planning stage.

Design has been recognized to be essential to experiments for a considerable time, and the editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned unrefereed.

i) Discussion should cover implications and consequences and not just recapitulate the results; conclusions should also be summarized.
j) There should be brief acknowledgments.
k) There ought to be references in the conventional format. Global Journals recommends APA format.

Authors should carefully consider the preparation of papers to ensure that they communicate effectively. Papers are much more likely to be accepted if they are carefully designed and laid out, contain few or no errors, are summarizing, and follow instructions. They will also be published with much fewer delays than those that require much technical and editorial correction.

The Editorial Board reserves the right to make literary corrections and suggestions to improve brevity.

FORMAT STRUCTURE

It is necessary that authors take care in submitting a manuscript that is written in simple language and adheres to published guidelines.

All manuscripts submitted to Global Journals should include:

Title

The title page must carry an informative title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) where the work was carried out.

Author details

The full postal address of any related author(s) must be specified.

Abstract

The abstract is the foundation of the research paper. It should be clear and concise and must contain the objective of the paper and inferences drawn. It is advised to not include big mathematical equations or complicated jargon.

Many researchers searching for information online will use search engines such as Google, Yahoo or others. By optimizing your paper for search engines, you will amplify the chance of someone finding it. In turn, this will make it more likely to be viewed and cited in further works. Global Journals has compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

Keywords

A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, "What words would a source have to include to be truly valuable in a research paper?" Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

Numerical Methods

Numerical methods used should be transparent and, where appropriate, supported by references.

Abbreviations

Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

Formulas and equations

Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

Tables, Figures, and Figure Legends

Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.

Figures

Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

PREPARATION OF ELETRONIC FIGURES FOR PUBLICATION

Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/ photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution at final image size ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs): >350 dpi; figures containing both halftone and line images: >650 dpi.

Color charges: Authors are advised to pay the full cost for the reproduction of their color artwork. Hence, please note that if there is color artwork in your manuscript when it is accepted for publication, we would require you to complete and return a Color Work Agreement form before your paper can be published. Also, you can email your editor to remove the color fee after acceptance of the paper.

TIPS FOR WRITING A GOOD QUALITY MEDICAL RESEARCH PAPER

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.

6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.

Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- o Explain the value (significance) of the study.
- o Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- o Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- o Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- o Report the method and not the particulars of each process that engaged the same methodology.
- o Describe the method entirely.
- o To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- o Simplify—detail how procedures were completed, not how they were performed on a particular day.
- o If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- o Resources and methods are not a set of information.
- o Skip all descriptive information and surroundings—save it for the argument.
- o Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- o Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- o In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- o Present a background, such as by describing the question that was addressed by creation of an exacting study.
- o Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- o Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- o Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- o Do not include raw data or intermediate calculations in a research manuscript.
- o Do not present similar data more than once.
- o A manuscript should complement any figures or tables, not duplicate information.
- o Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- o You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- o Give details of all of your remarks as much as possible, focusing on mechanisms.
- o Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- o One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- o Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

Segment draft and final research paper: You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

Written material: You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.

Please note that following table is only a Grading of "Paper Compilation" and not on "Performed/Stated Research" whose grading solely depends on Individual Assigned Peer Reviewer and Editorial Board Member. These can be available only on request and after decision of Paper. This report will be the property of Global Journals.

Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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