



# Regulation of DNA Methylation in Female Subjects with Increased (Pathological) Level of Circulating Immune Complexes and Breast Tumours

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## Abstract

**Background:** Pathological effects of circulating immune complexes have not been extensively explored, in the fields of oncology, considering its potentials to mediate immuno-pathological effects. The purpose of this study was to assess DNA methylation patterns in breast tumours and apparently healthy female subjects with increased levels of circulating immune complexes.

**Methods:** We assayed serum samples from 50 apparently healthy female volunteers, 49 subjects with benign and malignant breast tumours (24 with benign tumour and 25 with malignant tumour) for circulating immune complexes using Polyethylene Glycol (PEG) immunoprecipitation, Isolated cell free DNA (FitAmp blood kit (Epigentek, USA) was assayed for global decrease or increase in 5-methylcytosine (5-mC) using immunoassay (Colorimetric) method.

**Results:** The proportion of subjects with benign and malignant tumours demonstrating increased level of CIC was 70.8% (17 of 24 subjects) and 96% (24 of 25 subjects) respectively and 32% (16 of 50 subjects) in apparently healthy subjects. Between DNA methylation aberration markers, the frequency of hypomethylation and unmethylation were significantly high in benign, malignant and healthy subjects with increased level of circulating immune complexes. Expression of normal methylation was significantly high in healthy control subjects with normal level of circulating immune complexes. Strong positive correlation was obtained between levels of immune complexes and DNA methylation shifts  $P=0.000$ .

**Conclusion:** DNA methylation aberration (hypomethylation and unmethylation) in the global CpG regions were synonymous with increased level of circulating immune complexes. This revealed possible pathological effect of immune complexes on DNA methylation patterns in healthy and in diseased conditions.

## Introduction

Deoxyribonucleic acid (DNA) methylation is the covalent addition of methyl group at the 5-carbon of the cytosines linked by phosphodiester bond to guanines (the CpG dinucleotides) to form 5-methylcytosine (5-mC). Overall, mammalian genomes are depleted of CpG sites that may result from the mutagenic potential of 5mC that can deaminate to thymine [1,2], but rather show a tendency to concentrate at specific clusters, called CpG islands. The remaining CpG sites are spread out across the genome where they are heavily methylated (the global DNA methylation) with the exception of CpG islands [2]. DNA methylation is essential for silencing retroviral elements, regulating tissue-specific gene expression, genomic imprinting, and X chromosome inactivation. Importantly, DNA methylation in different genomic regions may exert different influences on gene activities based on the underlying genetic sequence [2]. Usually, only a very small amount of CpG island promoters are methylated in normal cells. When they are hypermethylated, it is usually in the

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context of tumorigenesis or abnormal development, in this case, the global genome CpGs are lowly methylated [2].

An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence [3]. Epigenetic mechanisms have now emerged as key contributors to the alterations of genome structure and function [4]. Among the three pillars of epigenetic regulation vis-a-vis DNA methylation, histone modifications, and non-coding Ribonucleic Acid (RNA) species [4], we looked at the DNA methylation patterns because it is the most widely studied epigenetic mechanism. Alterations of these epigenetic mechanisms affect the vast majority of nuclear processes, including gene transcription, expression and silencing, DNA replication and repair, cell cycle progression, and telomere and centromere structure and function [4], in which case tumorigenesis may ensue. Epigenetic cell alterations could emanate from the environment and triggers an intracellular pathway. Thus, in this study, we considered Circulating Immune Complexes (CIC) which is exogenously and endogenously sourced and systemically generated antigen-antibody complex due to immune response, a possible inducer of DNA methylation aberration. Circulating Immune Complexes have the capability of inducing intracellular activities, thus mediating inflammation [5]. The ability of CICs to persist in circulation due to antigenic supplies from the environmental cues would result to chronic inflammation [5]. This study considered the clinical importance of antigenic retention and formation of complex with its corresponding antibody as well as its possible persistence, as potential inducer of physiological stress that may lead to patho-physiological and immunological phenomena, thus, altering normal DNA methylation patterns. Extensive work has been done on influential role of immune complex deposition and kidney damage [6,7]. But little or no consideration has been given to the possible influence the persistence and deposition of immune complexes may have on normal epigenetic regulation, thus causing alteration of normal cell epigenetic process, which may result to development of tumour microenvironments and tumorigenesis, in any organs of the body other than the kidney [6,7].

Some immunological mechanisms by which immune complexes would persist has been shown in mice and may include, parasite-specific antibody responses induced by infection of mice with *Plasmodium chabaudi* which contain short and long-lived components as well as memory B cells responsible for a faster antibody response during re-infection [8]. Furthermore, parasite-specific antibodies to the C-terminal fragment of merozoite surface protein-1 (MSP-1) undergo avidity maturation. However, antibodies with both low and high avidity persist throughout infection and after re-infection, suggesting repeated rounds of activation and maturation of memory B cells [8]. In any of these examples exogenous or endogenous antigens circulate in the blood for long periods, enabling chronicity, initiating chronic inflammation.

The pathological mechanism of immune complexes, involves the combination of immunoglobulin M (IgM) or immunoglobulin G (IgG) antibodies with antigen, subsequent activation of the complement cascade, generating active peptides such as Complement 5a (C5a), which, in addition to dilating capillaries and increasing vascular permeability, contracts smooth muscle and mobilizes phagocytic cells [5]. Binding of immune complexes to neutrophils and macrophages also activates the respiratory burst with generation of toxic oxygen products (Reactive Oxygen Species) such as hydrogen peroxide, hydroxyl radical, hypochlorous acid, and chloramines.

Lysosomal proteolytic enzymes released by neutrophils in response to binding to immune complexes, together with toxic oxygen products, produce a potent system that can damage protein and lead to blood vessel damage with haemorrhagic necrosis and further local tissue destruction (a source of damage associated molecular pattern) and with this, more immune complexes are being formed. After an insult to tissues, pro-inflammatory stimuli and adhesion molecules presented by the inflamed endothelium rapidly induce neutrophil rolling and adhesion to the vessel wall and subsequent transmigration into tissue [9]. Resting neutrophils can also acquire a state of pre-activation that amplifies their responsiveness to subsequent external stimuli in a process referred to as priming. Both localizes and augments the neutrophil response during inflammation [10]. Fundamental to an appreciation of the role of neutrophils in immune complex-induced injury is the principle that the arsenal of weapons that they have evolved to destroy microorganisms also play a significant role in damaging tissue. Engagement of Fragment crystallisable receptors (FcRs) and complement receptors triggers phagocytosis, release of enzymes and vasoactive amines, and generation of a vigorous oxidative burst [5]. We considered that under such physiological stress, epigenetic cell alterations could as well occur due to possible oxidative induced cell epigenomic perturbation. Furthermore, research has revealed high rate of abnormal methylation patterns (hypomethylation, unmethylation and hypermethylation) in subjects living in high environmentally polluted areas in Nigeria [11]. Thus extension of research tracing or linking the pathological effects of CIC to tumorigenesis or carcinogenesis is of paramount importance in the quest for cancer prevention and solution to its poor prognosis.

Thus, we considered that if CIC could persistently accumulate in the system, components of immune complexes may advance the level of expression of pro-inflammatory molecules and DNA oxidative marker owing to the fact that Immune complex accumulation leads to a broad spectrum of proinflammatory effects, including complement activation with release of phlogistic C3a and C5a peptides and cytokine secretion from FcγR-expressing cells [12]. Some studies showed evidence that chronic inflammation is linked to breast cancer recurrence and that elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients [13]. In addition, experimental studies clearly indicate that inflammatory mediators promote tumour development in cancer prone animal strains. This chronic inflammatory response to persistent CIC increases cancer risk both directly, through DNA damage, and indirectly, through tissue remodelling and fibrosis [14]. This DNA damage may occur as epigenetic cell alteration by obstruction of the methyl group attachment process in DNAs. Thus the connection between environmental influences and epigenetic alterations has prompted the need to evaluate methylation status along with levels of expression of circulating immune complexes.

This study is prompted to access DNA methylation shifts from the perspective that persistent immune circulation can induce chronic inflammation and that the role of inflammation in the initiation and progression of cancer is as a result of persistent or chronic inflammatory responses in the context of persistent tissue injury thus mediating cell transformation through DNA damage. On the other hand, tumour cells produce pro-inflammatory factors that encourage chronic inflammation and tumour growth [15,16]. In whichever way, immune complexes are indicted in the inflammatory response. Thus justifying the assessment of DNA methylation shifts in subjects harbouring what we referred to as pathological or increased levels of

immune complexes.

## Material and Methods

We recruited Ninety nine (99) female subjects for this study. These women were grouped into 49 subjects with breast tumours (benign and malignant) and 50 apparently healthy subjects. The subjects with breast tumours were sub grouped into 24 female subjects with benign breast tumour and 25 female subjects with malignant breast tumour (Cancer subjects). The subjects with benign and malignant breast tumours were attending clinic at the surgical unit of Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra State. The diagnosis of breast tumour was established by histopathological examination of biopsy and detection of tumour associated antigen 15-3 (CA 15-3). The 50 apparently healthy control subjects were confirmed free from breast tumours by clinical examination using physical breast examination by Surgeon [17] and exclusion of tumour associated antigen 15-3 (CA 15-3). None of the subjects had received any form of treatment (chemotherapy, surgery, radiotherapy or immunotherapy) for breast tumour prior to the study. All the subjects were screened clinically and biochemically to exclude any autoimmune diseases and Human Immunodeficiency Virus. Approval for the study was obtained from the ethics committee of Nnamdi Azikiwe University Teaching Hospital. Informed consent was obtained from the subjects before participation. All the subjects were administered questionnaire to obtain medical history and demographic information.

### Specimen collection

Ten (10) ml of fasting blood sample was drawn by veni-puncture from all the subjects. The blood samples were allowed to clot in a plain vacutainer tube at room temperature, for 30 min. The retracted clot was removed by centrifugation (Sorvall RC5C HS-4 rotor at 1500 xg for 15 min) at room temperature and the formed serum was carefully pipetted into another tube. Criteria for blood sample collection were made to suit the various parameters required to be tested in this study. Immune complex precipitation, dissociation DNA extraction and CA15-3 antigen detection from the serum were carried out immediately. The remaining sera were stored at -20°C.

### DNA isolation

We used *FitAmp* blood kit (Epigentek, USA) as described by the manufacturer to isolate tiny amounts of DNA from micro-dissection samples serum. For each 500 µl of serum sample, we added 20 µl of Epigentek protease and 500 µl of DNA isolation buffer (FA3) and mixed very well, incubated at 65°C for 10 min. We transferred 500 µl of the mixture to an Epigentek spin column in a 2 ml collection tube and centrifuged at 12,000 rotors per minute (rpm) for 30 sec. The flow through was discarded and the column replaced to the collection tube and the remaining volume of mixture was transferred to the column, centrifuged again at 12,000 rpm for 30 sec. The flow through was again discarded and the column replaced to the collection tube.

We added 300 µl of 70% ethanol to the spin column and centrifuge at 12,000 rpm for 20 sec. The flow through was discarded and the column replaced to the collection tube. We then added 200 µl of 90% ethanol to the column and centrifuge at 12,000 rpm for 20 sec. The flow through was discarded and the column replaced to the collection tube. We added another 200 µl of 90% ethanol to the column and centrifuged at 12,000 rpm for 40 sec. We placed the column in a new 1.5 ml vial and added 18 µl of Epigentek DNA Elution Solution and centrifuged at 12,000 rpm for 20 sec to elute DNA. The eluted DNA was stored at -20°C and the analysis was carried out within one week

of storage. DNA isolated from the serum of 50 healthy subjects served as normal controls for the assessment of abnormal methylation.

## Methods

### Immune complex precipitation and estimation

Approximately 0.2 ml (1 part) of the freshly obtained serum was mixed with 0.4 ml (2 parts) of 0.01 M-borate buffer, pH 8.4. To this mixture, 27 parts of 4.166% PEG was added (final 1:30 serum dilution and 3.75% PEG concentration was obtained) (Brunner and Sigal, 2000). The mixture was incubated at room temperature for 60 minutes; the turbidity developed was measured spectrophotometrically at 450 nm against control containing 1:30 diluted serum in borate buffer without PEG. The level of CIC in serum was expressed in terms of OD450 measured at the end of 60 min. The result was expressed as Peg Index derived by the formula: Peg Index = OD450 with Peg - OD450 with Borate Buffer Saline (BBS) without PEG x 1000 [18]. Mean + 2 SD (at 95% confidence interval) method, was used to determine the reference values of Circulating immune complex in 100 healthy subjects [19]. Value of 56.7 ugEq/ml was regarded as cut off values. Values above 56.7 ugEq/ml were regarded as high.

### Methylated DNA quantification

We assayed the methylation status of the global CpG in circulating DNA, based on the ability of DNA to bind to strip wells that are specifically treated to have a high DNA affinity, thus enabling the methylated fraction of DNA to be detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of methylated DNA is proportional to the Optical Density (OD) intensity measured. The calculated quantity was analysed to determine the actual methylation status (hypomethylation, unmethylation, hypermethylation and normal methylation).

The procedure was as directed by the manufacturer (Epigentek, USA). Briefly, DNAs were bound to the wells using eighty micro-litre (80 µl) of binding solution. This was followed by 1 µl of unmethylated and methylated DNAs as negative and positive controls we then added 20 µl (100 ng) of the isolated DNA sample. The solutions were mixed by shaking the plate on the bench several times, ensuring that the solutions coat the bottom of the well evenly. The strip plate was covered with plate seal and incubated at 37°C for 90 min. The binding reaction solution was removed from each well and the wells were washed three times with 150 µl of diluted wash buffer. We applied 50 µl of the diluted capture antibody (1:1000 dilution) with diluted wash buffer to each well, then covered and incubated at room temperature for 60 min. After the removal of the diluted capture antibody solution. The wells were washed three times with 150 µl of diluted wash buffer. We applied 50 µl of the diluted detection antibody (1:2000 dilution) to each well, then covered and incubated at room temperature for 30 min. the remaining diluted detection antibody solution was remove from wells, and the wells were washed four times with 150 µl of diluted wash buffer). We applied 50 µl of the diluted enhancer solution (1:5000) dilution with the diluted wash buffer. Afterwards, 50µl of the diluted enhancer solution was added to each well, the wells were covered and incubate at room temperature for 30 min. The diluted enhancer solution was removed from each well and the wells were washed five times with 150 µl of diluted Wash Buffer). We developed the plate using 100 µl of developer solution and incubated the plate at room temperature for 10 min away from light. Colour change was monitored in the sample wells and control wells. Then

**Table 1:** Subjects with Malignant, Benign Breast Tumours and Apparently Healthy Subjects with Increased and Normal Levels of Circulating Immune Complexes.

Subjects	Frequency of CIC	P value
Benign Tumour (N=24)	CIC <sup>+</sup> =17(70.8%)	P=0.000
	vs. CIC <sup>-</sup> =7 (29.2%)	
Malignant Tumour (N=25)	CIC <sup>+</sup> =24 (96%)	
	vs. CIC <sup>-</sup> =1 (4%)	
Healthy Subjects (N=50)	CIC <sup>+</sup> =16 (32%)	P=0.000
	vs. CIC <sup>-</sup> =34 (68%)	

the reaction was stopped using 100 µl of stop solution when colour in the positive control wells turned medium blue. The solution was mixed by gently shaking the frame on the bench and waited for 2 min to allow the colour reaction to be completely stopped. The colour change to yellow after adding stop solution and the absorbance was read on a microplate reader at 450 nm within 2 min to 15 min.

5-mC calculation and relative quantification.

To determine the relative methylation status of two different DNA samples, calculation of percentage of 5-mC in total DNA was carried out using the following formula.

$$5-mC\% = \frac{(Sample\ OD - ME3\ OD) \div 100 \times 100}{ME4\ OD - ME3\ OD \times 2 \div 5}$$

2: is a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC.

S= the amount of input sample DNA in ng = 100ng

P= the amount of input positive control in ng = 5ng

Positive control is a methylated polynucleotide containing 50% of 5-methylcytosine

Negative control is an unmethylated polynucleotide containing 50% of cytosine

## Statistical Analysis

SPSS version 23 (IBM Inc., USA) was used to analyze the data. Descriptive statistics were performed for distribution patterns of DNA methylation between subjects with increased and normal levels of circulating immune complexes. Mean +2SD (at 95% confidence interval) method was used to determine the reference values in healthy subjects [19]. The P values lower than 0.05 were considered statistically significant. Chi-square was used to determine the significant association of Nominal variables.

## Results

### Subjects with Malignant, Benign Breast Tumours and Apparently Healthy Subjects with Increased and Normal Levels of Circulating Immune Complexes

Out of 24 subjects with benign tumour, 17 (70.83%) had increased level of immune complexes while 7(29.16%) had normal level of circulating immune complexes. Out of 25 subjects with malignant tumour, 24(96%) had increased level of immune complexes while 1 (4%) had normal level of immune complexes. Out of 50 apparently healthy subjects used as control, 16(32%), had increased level of immune complexes while 34 (68%) had normal level of circulating immune complexes (Table 1).

### Distribution of DNA Methylation Patterns in Subjects with Tumours and Apparently Healthy, Expressing increased and Normal Levels of Circulating Immune Complexes

We detected the levels of 5mC in the global CpG of the circulating DNAs to ascertain the levels of global DNA methylation shifts in subjects with breast tumours (benign and malignant) and apparently healthy subjects. Our analysis showed that in 24 subjects with benign breast tumour, out of the 17(70.8%) subjects with increased level of CICs, 6(35.3%) had hypomethylation, 1(5.9%) had hypermethylation, 6(35.3%) had unmethylation, while 3(17.4%) had normal methylation. On the other hand, out of the 7(29.2%) subjects with normal level of CICs, 1(14.3%) had hypomethylation, 1(14.3%) had hypermethylation, 2(28.6%) had unmethylation while 3(42.9%) had normal methylation (Table 2).

In 25 subjects with malignant tumour, 24 subjects had increased level of CICs. Out these proportion, 11(45.8%) had hypomethylation, 4(16.7%) had hypermethylation, 8(33.3%) had unmethylation while non had normal methylation.

In 50 apparently healthy subjects, 16(32%) had increased level of CICs and out this proportion, 4(25%) had hypomethylation, non had hypermethylation, 3(18.8%) had unmethylation while 9(56.3%) had normal methylation. On the other hand, out of the 34(68%) with normal CICs, 1(2.9%) had hypomethylation, non had hypermethylation, 2(5.9%) had unmethylation while 31(91.2%) had normal methylation. The result also indicated strong positive correlation of levels of CIC with DNA methylation shift.

## Discussion

The formation of immune complexes and the activation of complement were associated with the appearance of a wide variety of tissue pathology in experimental animals, which cleared as the immune complexes were removed from the circulation. These pathologic effects included vasculitis, carditis, glomerulonephritis, as well as rheumatologic and dermatologic manifestations of disease [6]. In sequence to this, we observed increased level of CICs in subjects with benign and malignant breast tumours. Sequel to that, significant number of the subjects with increased level of CICs expressed high frequency of global DNA methylation aberration with high indication on hypomethylation and unmethylation. The high frequency of DNA methylation aberration was also seen in healthy subjects (subjects without tumours) that harbour increased levels of CICs. This is in conformity with earlier research, that cancer cells exhibit widespread loss of intergenic DNA methylation [20,21] with gain of methylation at many gene-associated CpG islands [20,21]. In normal cells the repetitive portion of the genome is heavily methylated while most CpG islands remain unmethylated [21]. Very little or nothing has been done in linking pathological effect of IC to this epigenetic alterations. This work highlighted on the possible DNA methylation aberration in the presence of increased level of CIC in subjects with tumours and apparently healthy subjects.

We opined that with 96% and 71% of the subjects with malignant tumour and benign tumour respectively, expressing high level of CIC, increased serum level of CIC could be marked as a prominent phenomenon in breast tumours. Research has shown that the constituents of immune complexes are also well-defined demonstrating that the isotype and subclass of antibody within the immune complex as well as the nature of the antigen itself all play key roles in pathology [6]. We therefore suggested that DNA methylation

**Table 2:** Distribution of DNA Methylation Patterns in Subjects with Tumours and Apparently Healthy Expressing increased and Normal Levels of Circulating Immune Complexes.

Subjects		Hypometh	Hypermeth	Unmeth	Normalmeth
Benign (N=24)	CIC <sup>^</sup> (N=17) vs.	7 (41.2%)	1 (5.9%)	6 (35.3%)	3 (17.4%)
	CIC <sup>v</sup> (N=7)	1 (14.3%)	1 (14.3%)	2 (28.6%)	3 (42.9%)
Benign (N=24) Malignant (N=25)	CIC <sup>^</sup> (N=17) vs.	6 (35.3%)	1 (5.9%)	6 (35.3%)	3 (17.4%)
	CIC <sup>^</sup> (N=24) CIC <sup>^</sup> (N=16) vs.	11 (45.8%) 4 (25%)	4 (16.7%) 0	9 (33.3%) 3 (18.8)	0 9 (56.3%)
Healthy (N=50)	CIC <sup>v</sup> (N=34)	1 (2.9%)	0	2 (5.9%)	31 (91.2%)

**Keys:** CIC<sup>^</sup>: Increased level of Circulating Immune Complexes; CIC<sup>v</sup>: Decreased or Normal level of Circulating Immune Complexes; Hypometh: Hypomethylation; Hypermeth: Hypermethylation; Unmeth: Unmethylation; Normal Meth: Normal Methylation

aberration among other stress induced abnormalities could be a product of physiological-oxidative stress due to persistence of CICs, which could mediate perturbation of epigenome or the DNA methylome. The word perturbation is considered suitable and it is in line with the fact that DNA methylation can also be altered by repeated modulation of the microenvironment [2].

Sequel to the possible oxidative stress that may ensue due to persistent expression of pro-inflammatory molecules in the presence of CICs, we deemed it wise to check the methylation status of the subjects in such a chronic inflammatory environment believing that persistence of CIC may be an enhancer of DNA damage. This hypothesis is supported by earlier research that the reactive oxygen species (ROS) associated with chronic inflammation is another source of DNA damage with the potential to affect DNA methylation. Halogenated pyrimidines, one form of ROS-induced damage, mimic 5-methylcytosine and stimulate DNMT1-mediated CpG methylation *in vitro* and *in vivo* [21]. Indeed, study of the glutathione peroxidase 1 and 2 double knockout model of inflammatory bowel disease found that 60% of genes that are hypermethylated in colon cancers also exhibit aberrant methylation in the inflamed non-cancerous precursor tissues [22,23], indicating that inflamed condition other than the cancerous state, induce the aberrant methylation status. This is in line with our findings showing occurrence of DNA methylation aberration in healthy subjects (subjects without tumours) with increased level of CICs and normal DNA methylation pattern in healthy subjects with normal level of CICs. Besides, we have earlier reported increased DNA methylation status in subjects found in proven environmentally polluted areas [11].

On detection of decreased and increased rate of addition of 5mC to the global CpG of the DNA, by the analysis of the methylation pattern in the subjects with increased and perhaps persistent level of CIC, we infer that increase in CIC level, due to its dynamic role in immunological activations may have adverse influential impact on methylation processes, during which addition of methyl group to the global genome CpG could be altered. This is because significant higher proportion of the subjects with increased levels of CICs had DNA methylation aberration such as hypomethylation and unmethylation compared to the same situation in subjects with normal levels of CICs in the 3 major groups.

We suggest that global DNA methylation alteration (hypomethylation and unmethylation) could be a marker for early diagnosis involving patho-physiological conditions, tumour microenvironments, tumourigenesis and cancer development. Research has shown that global decrease in 5-mC content of (DNA hypomethylation) is likely caused by methyl-deficiency due to a variety of environmental influences [1] and has been proposed

as a molecular marker in multiple biological processes such as cancer. It has been well demonstrated that the decrease in global DNA methylation is one of the most important characteristics of cancer [19]. Thus the quantification of 5-mC content or global methylation in cancer cells could provide very useful information for detection and analysis of this disease. We also suggest through the outcome of this study that increased level of CICs could be a serious pathophysiological phenomenon requiring adequate clinical attention and that such attention, if given, could help avert tumour development. Our suggestion points to the fact that increased persistent level of CICs could institute fundamental problems enhancing any patho-physiological condition, including increased expression of inflammatory molecules, perturbation of genome CpG methylation process and development of tumour microenvironment which may escalate to tumour and subsequently cancer.

## Conclusion

Immunological developments and its molecular products such as CICs, constantly formed, but not adequately cleared, could be a torn in the process of DNA methylation by enabling pathway to cell perturbation and subsequent aberrant epigenetic cell alteration which may result to DNA methylation shift.

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